TESTICULAR ANGIOGENESIS IN RATS - DEVELOPMENTAL CHANGES AND HORMONAL STIMULATION BY HUMAN CHORIONIC GONADOTROPHIN

by

CHUNG HOI SING (B. Med.)

A thesis submitted as partial fulfillment
of the requirement for the degree of
Master of Philosophy

July, 1998
Department of Physiology
Division of Basic Medical Sciences
The Chinese University of Hong Kong
Hong Kong

UL

TABLE OF CONTENTS

ABSTRACT

In the present study, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in endothelial cells and the quantification of blood vessel density by image analysis were used as indices of angiogenesis, and examined in rat testes after giving adult animals a single subcutaneous injection of 100 IU hCG and during the postnatal development from the age of 10- to 90-day old.

In response to hCG, a significant increase in the number of PCNA-positive endothelial cells per unit testis section area was found after 24 hours. By 48 hours post-injection, it reached a maximum of 30-fold above the control before declining to levels insignificantly different from basal at 5 days post-injection. The peak of PCNA-positive endothelial cells was followed by a significant increase in blood vessel density at 3-5 days post-hCG.

The hCG-induced endothelial cell proliferation was completely abolished in adult rats treated with ethane 1,2-dimethane sulphonate to destroy the Leydig cells. Chronic Leydig cell suppression by an 8-week treatment with exogenous testosterone released from subcutaneous silastic implants was without any significant effect on the hCG-induced increase in the number of PCNA-positive endothelial cells per unit intertubular area, despite a marked decrease in the level of vascular endothelial growth factor immunoreactivity in these cells. No differences could be found between the high (25 cm) and the low (3 cm) dose of testosterone implant that could or could not maintain near normal levels of spermatogenesis, respectively. Therefore it appears that hCG-induced endothelial cell proliferation is dependent on the presence of Leydig cells but less so on their levels of activities or the mass of the seminiferous tubules. Leydig cells are probably required, irrespective of their functional states, to mediate

the hCG-induced inflammation-like reaction in the testis and the inflammation mediators would then directly or indirectly stimulate endothelial cell proliferation.

Activation of testicular macrophages by intratesticular injection of polystyrene latex beads greatly enhanced the effect of hCG in inducing endothelial cell proliferation, and the actions of the two appeared to be synergistic. Depletion of testicular macrophages by intratesticular injection of liposome-entrapped dichloromethylene diphosphonate also potentiated the proliferative response of endothelial cells to the injection of hCG, though the effect was less when compared with macrophage activation. In the macrophage-depleted testes, the infiltration of polymorphonuclear (PMN) leukocytes accompanying the inflammation-like reaction to hCG was prolonged and remained evident 2 days post-hCG. These data suggest that testicular macrophages may provide a source of angiogenic factors or inflammation mediators to potentiate the hCG-induced angiogenic response in the testis, and in the macrophage-depleted state, the role of macrophages may be taken over by PMN.

During the postnatal development of the testis, the percentage of total testis section area occupied by intertubular tissues declined, and it was especially marked between the age of 20- and 30-day old. When the number of PCNA-positive endothelial cells was normalized against per unit intertubular area, the maximum values were found in the testes of 10- to 30-day old rats, which agreed with previous observations made in the rat, hamster and mouse using other techniques. This was later followed by a peak of blood vessel density (per unit intertubular area) in 50-day old rats.

摘 要

本實驗採用兩種實驗方法：(1)血管內皮細胞的增殖細胞核抗原（proliferating cell nuclear antigen）免疫組織化學染色法，和(2)影像分析軟件探測血管密度變化，作爲新生血管增殖的指標。運用以上兩種方法，觀察成年大白鼠對人絨毛膜促性腺激素（human chorionic gonadotrophin, hCG）一次性皮下注射引起的睪丸新生血管變化，以及大鼠出生後 10-90 日睪丸發育過程中新生血管的改變。

皮下注射 100 國際單位（IU）人絨毛膜促性腺激素 24 小時後，在睪丸橫切面單位面積內增殖細胞核抗原陽性的內皮細胞數目急劇上升。注射人絨毛膜促性腺激素後 2 天，內皮細胞增殖達到頂峰，是正常對照組的 30 倍，然後逐漸回落，注射後 5 天的數值明顯下降與對照組無顯著性差異。而睪丸血管密度則在注射後 3 和 5 天，出現顯著的增加。

當睪丸間質細胞（Leydig cell）被乙烷1，2二甲烷磺酸鹽（ethane dimethane sulphonate）選擇性殺滅，能夠完全消除注射人絨毛膜促性腺激素導致的內皮細胞增殖。儘管如此，當使用外源性的睪丸酮（testosterone）皮下包埋 8 周導致大鼠睪丸間質細胞功能抑制，睪丸間質單位面積內 hCG 導致的內皮細胞增殖未見顯著性改變。但睪丸間質細胞上血管內皮生長因子（vascular endothelial growth factor）的免疫活性有明顯的下降。高劑量（25cm）的睪丸酮皮下包埋可維持正常水平的精子形成，而低劑量（3cm）則不能維持，兩者所引起的人絨毛膜促性腺激素的內皮細胞增殖沒有顯著性差異。這些結果提示人絨毛膜促性激素導致的內皮細胞增殖依賴於睪丸間質細胞的存在，基本上與該細胞的活性水平和生精小管的成份無關。在人絨毛膜促性腺激發的睪丸類免疫反應中，睪丸間質細胞可能是必需的，且不受其功能狀態影響，免疫介質隨後直接或間接刺激內皮細胞的增殖。

雖然在睪丸內注射植物乳珠（latex beads）能激活巨噬細胞，大大增加人絨毛膜促性腺激素，導致的內皮細胞增殖效應，兩者似有協同效應。相反睪丸內注射微脂粒包裹的二氯甲烷二磷酸鹽（liposome-entrapped dichloromethylene diphosphonate）殺滅睪丸巨噬細胞，也激發 hCG 導致的內皮細胞增殖。相比巨噬細胞激活的實驗，此效應較爲遜色。在消除巨噬細胞的睪丸，多形核粒細胞滲入組織間隙的免疫反應時間延長至 hCG 注射後 2 日。這些結果表明，睪丸巨噬細胞也可能是一個血管增殖因子或免疫介質的來源，參與睪丸內 hCG 導致的血管增殖，在巨噬細胞消滅的情況下，巨噬細胞的作用可能被多形核粒細胞取代。

本實驗還報告了大白鼠出生後睪丸發育生長的過程中，睪丸間質佔睪丸橫切面面積的比率持續下降，在 20 日和 30 日兩組尤爲顯著。若考慮睪丸間質面積比率改變的影響，10 日至 30 日大白鼠睪丸血管內皮細胞增殖呈現取高值，此結果與過去在大白鼠、倉鼠和小白鼠的實驗是一致的。而 50 日組睪丸血管密度達到頂峰。

ACKNOWLEDGMENT

Like a pregnant woman in childbearing, I finally give birth to my thesis with sweat and joy.

I would like to express my sincerest gratitude to my supervisor, Dr. C.L. Au, whose invaluable guidance, patience and advice has made a reality of this thesis during my study period. I am also grateful to Professor P.Y.D. Wong (former Chairman) and Professor M.S.C. Tam (Acting Chairman) for kindly allowing me to work and use the facilities in the Department of Physiology.

I owe special thanks to Mrs. Aster L.F. Yee-Chan for her technical assistance in both experiments and computer works. Thanks also for useful advice and discussion from Dr. Andrew Laslett, Mr. Benny C.C. Sy, Miss W.S. Tang, Dr. C.C. Wong, Mr. Eugene Y.K. Wong, and Dr. M.S. Leung.

My heartful thanks to my wife Anna whose deep support, understanding and tolerance has made a reality of this thesis and our son. Actually I got my son at the end of July, 1998.

Finally, my Lord Jesus Christ has shown me such love and has given me such unfailing encouragement and such bright hopes in his grace. To God be glory for ever and ever.

1. Introduction

1.1 Angiogenesis in general

1.1.1 The concept of angiogenesis

Angiogenesis is defined as the formation of new blood vessels from pre-existing vessels. This is one of two recognized processes essential for the establishment and maintenance of vasculature (see reviews by Auerbach & Auerbach, 1994; Folkman, 1995b; Bicknell, Lewis & Ferrara, 1997; Norrby, 1997; Pepper, 1997; Risau, 1997). The other process - vasculogenesis, is used to describe the formation of early vascular plexus from mesoderm by the differentiation of primitive precursor cells called angioblasts and it occurs during embryogenesis. After the developing embryo has formed a primary vascular plexus, further blood vessels are generated predominantly by angiogenesis.

While vasculogenesis is limited to early embryogenesis, angiogenesis occurs not only in embryos but is an important component of tissue growth and development during postnatal life. However in adults, angiogenesis rarely occurs under physiological conditions except in the female reproductive system where it forms an integral part of follicular development, corpus luteum formation, repair of the endometrium during the menstrual cycle, formation of the placenta and development of the breast during pregnancy. Angiogenesis in adults is most commonly associated with pathological conditions such as wound healing, inflammation, diabetic retinopathy, psoriasis, rheumatoid arthritis, and solid tumor growth (see reviews by Folkman, 1995a; Breier et al, 1997; Pepper, 1997).

1.1.2 The process of angiogenesis

Although the exact molecular mechanisms responsible for angiogenesis are not known, the cascade of events leading to the formation of new vessels has been well documented (Hanahan & Folkman, 1996; Hanahan, 1997; Risau, 1997). The vascular growth entails either endothelial sprouting or intussusception. However, microvessel growth through sprouting is the predominant type and it develops in the following sequence of events. First, there is a local proteolytic degradation of the basement membrane and extracellular matrix surrounding the existing vessel. Secondly, there is a chemotactic migration of endothelial cells towards the site of angiogenic stimulus. As the endothelial cells migrate, they divide and multiply in numbers. A new blood vessel begins to take its form when the lumen formation begins either by curvature of the endothelial cell or vacuole formation in the cell. Finally, there is the synthesis of new basement membrane for anchoring the new blood vessel (see reviews by Auerbach & Auerbach, 1994; Hanahan & Folkman, 1996). The main target vessels for angiogenic stimuli are the postcapillary venules, small terminal venules and capillaries (see reviews by Norrby, 1997; Pepper, 1997; Risau, 1997). Among the different steps involved in angiogenesis, the demonstration of endothelial cell proliferation is most commonly used to indicate the occurrence of this event *in vivo* based on histological examination of tissue sections. In addition, the angiogenic potential of tissues, cells or unknown factors can be tested based on their ability to induce endothelial cell proliferation or migration using *in vitro* assays. (Auerbach, Auerbach & Polakowski, 1991; Jain *et al.*, 1997).

In the past several years, the identification of a number of well-defined growth factors, the observations made from genetically manipulated mice, and the recognition of the importance of cell-cell and cell-substrate (e.g. extracellular matrix) interactions have greatly expanded our understanding of the regulation of angiogenesis. The

actions of a variety of polypeptide growth factors appear to be orchestrated in a complex sequence of steps that lead to the new blood vessel formation. The communication between the growing vasculature and the tissue parenchyma, as well as interactions among the cells in the vessel wall, all appear to influence vascular development and growth. (see reviews by Gasparini, 1996; Beck & D'more, 1997; Breier et al, 1997; Norrby, 1997).

1.2 Measurement of angiogenesis

Angiogenesis can be quantitatively or qualitatively measured using a number of different methods. Basically they rely on determining the events (e.g. a step in the angiogenic process) or products (e.g. increase in blood vessel density) of angiogenesis. Besides there are methods which are less invasive than others and thus are more suited for clinical use.

1.2.1 *In vivo* assays

The quantification of angiogenesis *in vivo* has been performed primarily by one of the following three approaches: (1) microcirculatory preparations in animals, mostly chick embryo and rodents; (2) vascularization into biocompatible polymer matrix implants; and (3) excision of vascularized tissues from animals or human (Jain *et al.*, 1997).

Microcirculatory preparations allow *in vivo* observation of new vessel formation under light microscopy. Based on the methods of preparation, they can be divided into three broad categories: (1) chronic transparent chambers (e.g. rabbit ear chamber; dorsal skinfold chamber in mice, rats, hamsters or rabbits; cranial windows in mice or rats; hamster cheek pouch window); (2) exteriorized tissue preparations

[e.g., hamster cheek pouch; mouse, rat, or rabbit mesentery; mouse or rat liver; chick chorioallantoic membrane (CAM); air sacs in mice or rats]; and (3) *in situ* preparations (e.g., corneal pocket or iris implant in the eye). With the development of computer-assisted image analyses and microvascular technology, considerable information regarding the structure and function of new vessels can now be obtained using these techniques.

Vascularization into matrix implants requires a polymer matrix (gel or sponge) containing a known amount of angiogenic factor(s) or cells, to be glued or attached to a vascular bed. The structure and function of new vessels penetrating the matrix can then be measured in a variety of ways.

With excised tissues, the parameters characterizing angiogenesis are measured with a variety of techniques after the vascularized tissue has been excised. The techniques for measuring morphologic parameters include light or electron microscopy of vascular casts, histological examination of tissue sections stained for endothelial cells/basement membrane or perfused with intravascular markers (e.g., colloidal carbon, India ink, radioactively labelled red blood cells, high-molecular-weight tracers). Since endothelial cell proliferation is an essential step in angiogenesis, it has been most commonly used as an index of angiogenesis. There are a number of ways to identify proliferating endothelial cells (Woosley, 1991), and these include: (a) stereological measurement of increased endothelial cell number in the tissue; (b) immunohistochemical staining of proliferating endothelial cells using markers such as proliferating cell nuclear antigen (PCNA) and Ki67 (Linden, *et al.*, 1992); (c) mitotic figures; and (d) incorporation of tritiated thymidine or bromodeoxyuridine during DNA synthesis. However, the histological methods may be replaced by various clinically applicable and noninvasive techniques, such as

magnetic resonance imaging (MRI), functional computed tomography (FCT) and positron emission tomography (PET), as the spatial resolution of the images generated using these techniques improves. Angiogenesis *in vivo* can also be assessed qualitatively through the measurement of the plasma or urine concentration of angiogenic factors (Cockerill, Gamble & Vadas, 1995; Jain *et al.*, 1997).

1.2.2 *In vitro* assays

Significant insight into understanding the molecular and cellular biology of angiogenesis has come from *in vitro* assays using cultured endothelial cells from micro- or macrovessels. Although most angiogenic steps can be studied *in vitro*, research has been focussed on the migration and proliferation of endothelial cells. Limited research has been conducted on the dissolution of matrix and differentiation of vessels. A sandwich-culture assay has recently been adapted to the study of morphogenesis of blood vessels *in vitro* in a defined oxygen gradient. The migration studies primarily utilize the Boyden chamber or another variant of the under-agarose assay. The proliferation studies are based on cell counting, thymidine incorporation or staining techniques for cell proliferation (e.g. PCNA) (Auerbach, Auerbach & Polakowski, 1991; Jain *et al.*, 1997). Culture conditions for the induction of human angiogenesis *in vitro* using fragments of human placental blood vessel have recently been established. Once this novel assay has been validated, it will then be possible to examine its ability to detect and screen potential inhibitors and enhancers of human angiogenesis (Brown *et al.*, 1996).

Accurate and reliable quantification of the angiogenic response, both *in vivo* and *in vitro*, is an essential requirement for the study of new blood vessel growth. Over many years, ingenious ways have been developed for measuring this process,

and they have contributed much to our present understanding of mechanism of angiogenesis.

1.3 Angiogenic factors

Since the first isolation of an angiogenic factor from tumors in the early 1970s, at least 12 angiogenic factors have been found to stimulate angiogenesis (Folkman & Klagsbrun, 1987; Bussolino *et al.*, 1996; Beck & D'more, 1997; Iruela-Arispe & Dvorak, 1997). Among these angiogenic factors, basic fibroblast growth factor (bFGF) is the first to be identified, and it has also been used as a prototype for establishing a number of criteria that characterize angiogenic growth factors in general. These include their ability to stimulate endothelial cell proliferation *in vitro* or to induce angiogenesis *in vivo*, and their localization at the sites of vessel growth (Iruela-Arispe & Dvorak, 1997).

Although there are many factors which are known to stimulate angiogenesis, majority of them are angiogenic only *in vivo*. This has led Folkman and Haudenschild (1980) to propose a distinction between direct and indirect angiogenic factors. Direct factors would be those that could elicit angiogenesis, endothelial cell migration/proliferation both *in vitro* and *in vivo*. Indirect factors are those that cannot induce angiogenesis on their own *in vitro* but can do so only *in vivo* by inducing other cells to secrete direct angiogenic factors.

Among the direct angiogenic factors are the acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). They act directly on endothelial cells and are potent stimulators of their proliferation/migration *in vitro*. However, among these few factors, only VEGF has the signal peptide for secretion and also its

mitogenic action is specific only to endothelial cells (Ferrara, 1996; Ferrara & Davis-Smyth, 1997). So it makes VEGF a very attractive candidate for the study of how the angiogenic process is regulated.

VEGF is a family of heparin-binding, homodimeric glycoproteins (Ferrara *et al.*, 1992; Dvorak *et al.*, 1995; Ferrara, 1996; Ferrara & Davis-Smyth, 1997). Complementary DNA sequence analysis of a variety of human VEGF clones has indicated that VEGF may exist as five different isoforms having 121, 145, 165, 189 and 206 amino acid residues ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). It has now been well established that alternative exon splicing of a single VEGF gene is the basis for this molecular heterogeneity. Molecular sizes of VEGF in rodents are shorter than the corresponding human species by one amino acid. Due to the presence of different isoforms, molecular weight of VEGF ranges from 34-56 kDa. All isoforms have the signal peptide for secretion. VEGF proteins may become available to endothelial cells by different mechanisms: as freely diffusible proteins ($VEGF_{121}$, $VEGF_{165}$) or after protease activation and cleavage of the longer isoforms ($VEGF_{189}$, $VEGF_{206}$, probably also $VEGF_{145}$) from their binding to heparin-containing proteoglycans on the cell surface, basement membrane or extracellular matrix. VEGF is mitogenic only to endothelial cells and it has direct action *in vitro* to stimulate their proliferation.

1.4 Angiogenesis in the female reproductive system

In adult tissues, angiogenesis normally occurs under pathological conditions such as during inflammation and tissue repair. The female reproductive system is the only exception where angiogenesis occurs physiologically as the tissues (i.e. ovarian follicle, corpus luteum, postmenstrual endometrium, pregnant uterus and mammary

gland) exhibit dynamic cyclical growth and regression (Reynolds, Killilea & Redmer, 1992; Modlich, Kaup & Augustin, 1996; Gordon *et al.*, 1995; Pepper, 1997; see review by Findlay, 1986). In these circumstances angiogenesis is tightly regulated to match the metablic demands of the tissues. Many diseases of the female reproductive system, including cancers of the female reproductive tissues, endometriosis and prolactinomas, also involve angiogenesis (McLaren, *et al.*, 1996; see review by Koos & LeMaire, 1983).

In the ovary, the development of the follicular capillary network may play a role in the selection of the dominant follicle, and after ovulation, the rapid vascularization of the corpus luteum is crucial to the production of progesterone. In concert with the changes in the ovary, angiogenesis within the endometrium must occur before implantation and placentation can successfully occur. These new vessels supply the nutrients needed for cell growth and biosynthesis in the embryo, and make possible the endocrine communication among the ovary, the hypothalamic-pituitary unit, and the uterus.

Ovarian, uterine and placental tissues have been shown to contain and produce angiogenic factors (Giudice, 1994; Cooper *et al.*, 1995; Gordon *et al.*, 1995; Gordon *et al.*, 1996). Recent evidence also indicates that angiogenesis in the female reproductive system is likely to be under the control of the female sex steroids (estrogens and progesterone) and gonadotrophins (particularly LH/hCG) (Charnock-Jones *et al.*, 1993; Cullinan-Bove & Koo, 1993; Dissen *et al.*, 1994; Koos, 1995; Hyder *et al.*, 1996; Das *et al.*, 1997)

1.5 Evidence of hormonally-regulated angiogenesis in endocrine tissues

Numerous evidence has accumulated in the past which suggests that the process of angiogenesis in the female reproductive system is hormonally-regulated (Ferenczy, Bertrand & Gelfand, 1979; Sato, Ishibashi & Koide, 1982; Koos & LeMaire, 1983; Taraska *et al.*, 1989; Dissen *et al.*, 1994; Ferrara *et al.*, 1998). More recently, a lot of the attention has been directed towards examining the role of VEGF in mediating the hormonally-regulated angiogenesis. VEGF is not only a potent angiogenic factor which is mitogenic only to endothelial cells, it also occurs in many endocrine glands (e.g. testis, thyroid, pancreas, adrenal and pituitary) (Table 1.1) and hormone-responsive tissues like the uterus, fallopian tube, placenta and breast.

Table 1.1 Occurrence of VEGF in endocrine glands and hormone-responsive tissues

Location	Cell origin	References
pituitary	folliculo-stellate cells	Leung *et al.* (1989)
testis	Leydig cells, Sertoli cells	Au *et al.* (1997)
ovary	granulosa (cumulus) cells, thecal cells; luteal cells	Phillips *et al.* (1990) Kamat *et al.* (1995)
thyroid	follicular cells	Sato *et al.* (1995)
pancreas	islets β-cells	Kuroda *et al.* (1995)
adrenal	adrenal cortex	Shweiki *et al.* (1993)
uterus	glandular epithelial cells, stromal cells	Torry *et al.* (1996)
fallopian tube	luminal epithelium	Gordon *et al.* (1996)
placenta	macrophages, glandular epithelium, trophoblast	Cooper *et al.* (1995)
breast	lobular and ductal epithelium	Brown *et al.* (1995)

From those studies examining the involvement of hormones in regulating VEGF gene expression and/or secretion in some of these tissues (mainly the ovary, endometrium and thyroid), evidence has been accumulated strongly supporting the

possible role of VEGF in mediating hormonally-regulated angiogenesis. Presented below are the experimental findings indicating a role of trophic hormones in the regulation of VEGF expression, and hence angiogenesis, in the ovary and the thyroid gland.

1.5.1 Ovary

In the case of the ovary, the role of gonadotrophins in stimulating the expression of VEGF mRNA has been investigated using cultured luteinized granulosa cells (Neulen *et al.*, 1995) obtained from human ovarian follicles around the time of ovulation when there is a marked increase in follicular angiogenesis. hCG or LH was shown to be much more potent than FSH in stimulating VEGF expression in both a time- and a dose-dependant manner. Similarly, Laitinen *et al.* (1997) recently showed that recombinant human FSH (rhFSH) can induce an increase in VEGF mRNA levels in primary cultures of human granulosa-luteal (GL) cells when the cells were treated with rhFSH on days 5-7 of culture. Compared with the effect of hCG/LH, the delayed responsiveness to rhFSH was characteristic of this cell culture system. The time-course studies indicated that hCG induced a rapid increase in VEGF mRNA levels in cultured human GL cells, which was consistent with the results obtained with granulosa cells of human origin (Neulen *et al.*, 1995) and from rats (Koos, 1995). VEGF expression has also been examined in monkey corpora lutea by Northern blot analysis following GnRH antagonist treatment (Ravindranath *et al.*, 1992). VEGF mRNA levels in corpora lutea obtained from day 7 to 10 of the luteal phase were shown to be significantly depressed 3 days after treatment, suggesting that VEGF expression in corpus luteum is also dependent on gonadotrophin support. In another study, the suppression of gonadotropins release by the administration of a GnRH

antagonist significantly suppressed the increase of VEGF mRNA levels in the transplanted ovaries obtained from immature rats (Dissen *et al.*, 1994). The use of PMSG (pregnant mare serum gonadotropin) to bypass the suppression of endogenous FSH levels, failed to restore the VEGF mRNA levels in the transplanted ovary that were blunted by the LHRH antagonist. Finally, conditioned medium from gonadotrophin-treated human ovarian carcinoma cells was shown to be mitogenic to bovine endothelial cells, and this activity was blocked by neutralizing antibodies against LH or VEGF (Schiffenbauer *et al.*, 1997). Using RT-PCR, both LH and FSH were shown to produce a dose-dependent stimulation of VEGF expression in cultured human ovarian cancer spheroids. Based on above findings, it can be concluded that the stimulation of gonadotrophins, mainly LH/hCG, is involved in the regulation of VEGF mRNA levels in the ovarian follicles and corpora lutea, and thus may provide the basis for a hormonally-induced angiogenesis in the ovary.

1.5.2 Thyroid

Similar to the trophic effects of gonadotrophins on ovarian VEGF expression, in the thyroid gland, VEGF mRNA levels in cultured human thyroid follicles can be stimulated by thyroid stimulating hormone (TSH) (Sato *et al.*, 1995). The TSH effect can be mimicked by thyroid stimulating immunoglobulin (TSI) from Graves' disease patients, resulting in an even greater stimulation of VEGF mRNA levels than TSH. Furthermore a cell permeable analogue of cAMP (dibutyryl cAMP) can mimick the effects of TSH and TSI and produces a dose-related stimulation of VEGF expression in the same system of cultured human thyroid follicles (Sato *et al.*, 1995). Since TSI is an autoantibody against TSH receptor, and its binding to TSH receptor activates the same intracellular signalling transduction cascade (i.e. cAMP-protein kinase A

pathway) as that of TSH or this can be mimicked by exogenous cAMP analogues, thus the above data strongly support a role for TSH in the control of VEGF expression in the thyroid gland. Another study also showed that in three out of four thyroid cancer cell lines that were tested, TSH stimulated a significant increase in VEGF release as measured using ELISA (Soh *et al.*, 1996). Recently Viglietto *et al.* (1997) reported that VEGF mRNA expression was markedly enhanced in biopsies of goiters resected from Graves's patients. *In vivo* studies demonstrated that in the thyroid gland of thiouracil-fed rats, increased VEGF mRNA levels occurred subsequent to the compensatory rise in the serum TSH levels and in parallel with thyroid capillary proliferation. These data suggested that VEGF, released from thyrocytes in response to the chronic stimulation by TSH, may act through a paracrine mechanism to increase endothelial cell proliferation and hence blood vessel growth in the thyroid gland (Viglietto *et al.*, 1997). So in summary, the above data indicate that in response to TSH or TSI, normal thyroid cells as well as thyroid carcinoma cell lines can increase the VEGF expression. The combined results from *in vivo* and *in vitro* studies demonstrated that activation of the intracellular signalling transduction cascade linked to TSH binding to its receptors may trigger and maintain the dramatic hypervascularization of the thyroid gland through a coordinated regulation of VEGF expression in thyrocytes.

1.6 Angiogenesis in the testis

1.6.1 Structure of testicular vasculature

The vascular system of the testis possesses several peculiar anatomical features which indicate that it has important control over the function of the testis (see reviews by Free, 1977; Desjardins, 1989, 1993; Bergh & Damber, 1993; Setchell,

Maddocks & Brooks, 1994). These include a long unbranched testicular artery, the presence of a peritubular and an intertubular capillary network, the lining of the testicular vasculature by an unfenestrated endothelium and the failure of the capillaries to penetrate the seminiferous tubules. Due to the haemodynamic properties from a long unbranched vessel, testicular artery is considered to pose an upper limit on the rate of blood flow through the testis. The two testicular capillary networks have been proposed to offer an independent control of blood flow to the tubular and intertubular compartments of the testis. The peritubular capillaries vascularize the seminiferous tubules, whereas the intertubular capillaries preferentially supply and drain the testicular interstitium. An unfenestrated endothelium may impede the passage of important macromolecules like the gonadotrophins, from blood to the target cells within the testis. Finally the seminiferous tubules which made up the bulk of the testicular tissues and contain numerous actively dividing and differentiating germ cells, do not have a direct blood supply. Since oxygen has to diffuse from the capillaries in the intertubular areas to the seminiferous tubules, the centre of these tubules has been shown to be at the brink of hypoxia. In the view of the limitations imposed by the anatomical arrangement of the testicular vasculature, a well-developed and maintained blood supply appears to be extremely important to the normal activities within the testis and may provide the necessary local control of testicular function. In the vascular control within the testis or any other major organs, besides the acute effects from the alteration of the vascular permeability, and the regulation of blood flow by vasodilation or vasoconstriction, there is a medium- to long-term regulation by controlling the blood vessel formation or angiogenesis.

1.6.2 Angiogenic factors in the testis

It remains to be elucidated how the angiogenic process in the testis is initiated and controlled. Nevertheless, the two major somatic cell types within the testis, namely the Leydig cells and Sertoli cells, have been shown to produce or contain potent angiogenic factors (Table 1.2). Besides, the testis contains a resident population of white blood cells - macrophages, mast cells and lymphocytes, which may also play a role in the process of angiogenesis through their secretory products (Table 1.2 and 1.3).

Resident macrophages comprise up to 25% of the interstitial cell population in the testis of mammals including man, monkey, rat and boar (Kern *et al.* 1995). There are numerous reports demonstrating a significant interaction between Leydig cells and testicular macrophages within the testis (Miller, Bowman, & Rowland 1983; Bergh, 1985). This has led to the speculation that testicular macrophages are also involved in the process of angiogenesis within testis.

The macrophage is the major terminally differentiated cell type of the mononuclear phagocyte system. Its origin lies in the bone marrow. Once the monocytes migrate from the blood circulation into the extravascular tissues, they terminally differentiate into tissue macrophages, and remain resident in their organ sites. In general, macrophages also represent a cellular source of angiogenic factors. *In vitro* and *in vivo* studies have shown that macrophages produce a variety of angiogenic factors (Table 1.3) depending upon their state of activation (Sunderkotter *et al.*, 1991; Polverini, 1995). They can promote angiogenesis by two possible mechanisms: (a) influencing endothelial cell proliferation and differentiation, and/or (b) modifying the production of extracellular matrix components (Sunderkotter *et al.*, 1991; Leek, Lewis & Harris 1997).

Table 1.2 Angiogenic factors, cytokines and mediators of angiogenesis present in mammalian testes*

Substances	Species	Source/ localization	Detection method	Control of production or expression
EGF	Mouse	Sertoli cells	IHC	
bFGF	Rat	Sertoli cells	RPA, Western	FSH⇑
bFGF	Rat	Leydig cells	IHC, RPA	
PDGF	Rat	Leydig cells	IHC, RPA, Northern	hCG⇑
PDGF	Rat	Sertoli cells	RPA, bioassay, Northern	FSH⇓
TGFα	Rat	Sertoli cells	Northern, Western	
TGFα	Rat	Leydig cells	IHC	
TGFβ	Rat	Sertoli cells	RPA, Northern, Western, IHC	FSH⇓
TGFβ	Rat	Leydig cells	IHC	
TNFα	Rat	macrophages	Bioassay	LPS⇑
VEGF	human	Sertoli cells	Western, IHC, RT-PCR	
VEGF	human	Leydig cells	Western, IHC, RT-PCR	
AT-II	Rat	Leydig cells	RIA/HPLC	
IL-1	Rat	Sertoli cells	Bioassay	LPS⇑
IL-1	Rat	Leydig cells	Northern	IL-1β, LPS, hCG⇑
IL-1	Rat	Macrophages	Bioassay	
SP	human	Leydig cells	IHC	

* In part modified from Ergun *et al.* (1997); Gnessi, Fabbri & Spera (1997) and Norrby (1997).

Abbreviations for substances: EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; PDGF: platelet-derived growth factor; TGFβ: transforming growth factor β; TNFα: tumor necrosis factor α; AT-II: angiotensin-II; IL-1: interleukin-1; SP: substance P.

⇑ - stimulates or increases; ⇓ - inhibits or decreases.

HPLC: high pressure liquid chromatography; IHC: immunohistochemistry; RIA: radioimmunoassay; RPA: ribonuclease protection assay; RT-PCR: reverse transcriptase-polymerase chain reaction; Northern: Northern blot analysis; Western: Western blot analysis.

FSH: follicle stimulating hormone; hCG: human chorionic gonadotrophin; LPS: lipopolysaccharide.

Table 1.3 Macrophage-derived factors involved in angiogenesis

Mitogens
Basic Fibroblast growth factor (bFGF)
Epidermal growth factor (EGF)
Transforming growth factor-α (TGF-α)
Insulin-like growth factor I and II (IGF-I and -II)
Vascular permeability factor / Vascular endothelial growth factor
Migration inducing factors
Angiotropin
Human angiogenic factor (HAF)
Diverse angiogenic factors derived from macrophages
Angiotensin converting enzyme (ACE)
Granulocyte- and granulocyte-macrophages-colony stimulating factor (G-CSF and GM-CSF)
Inflammatory proteins
Interleukin 1(IL-1)
Interleukin 6 (IL-6)
Platelet-derived growth factor / thymidine phosphorylase (PDGF / TP)
Prostaglandins
Factors modulating the extracellular matrix
Collagenase
Elastase
Fibrin
Serine proteases
Plasminogen activator
Factors with complex effects
Transforming growth factor-β (TGF-β)
Tumor necrosis factor-α (TNF-α)

* Modified from Sunderkotter *et al.* (1991) and Leek, Lewis & Harris (1997)

So within the testis, angiogenic cytokines and other factors can be produced by a wide range of cell types, including Leydig cells, Sertoli cells, macrophages, mast cells, endothelial cells, and fibroblasts. These cells and their products may have complex interactions to control the angiogenic process.

1.6.3 Vascular effects of hCG/LH in the testis

The effect of hCG on the testicular vasculature has been well-documented. A single subcutaneous injection of 50-100 IU hCG in adult rats results in an initial decrease followed by an increase in testicular blood flow together with a change in the pattern of vasomotion (Setchell & Sharpe, 1981; Widmark, Damber & Bergh, 1986; Widmark *et al.*, 1987; Bergh, Damber & Widmark, 1988; van Vliet *et al.*, 1988). Accompanying the changes of blood flow, there is an inflammation-like increase in vascular permeability and polymorphonuclear leukocyte infiltration (Sharpe, 1979; Sharpe & Cooper, 1983; Bergh *et al.*, 1986, 1987; Widmark, Damber & Bergh, 1986; Widmark *et al.*, 1987). Similar responses may be induced in human testes (Hjertkvist *et al.*, 1993) and with the use of LH (Widmark, Damber & Bergh, 1989; Bergh, Damber & Widmark, 1990). Such vascular changes seen in the testis in response to LH/hCG are very similar to the inflammatory reaction and changes of blood flow in the ovulatory follicle exposed to the pre-ovulatory gonadotrophin surge (Espey, 1980; Gerdes *et al.*, 1992). Despite the similarity between the two events, no previous studies have attempted to examine whether the angiogenesis in the ovulatory follicles, which is most likely also linked to the gonadotrophin stimulation, could similarly be induced in the testis by LH/hCG.

1.6.4 Postnatal development of testicular vasculature

Although angiogenesis is equally important to the developing testis pre- and post-natally, the angiogenic process in the postnatal testis has not been well examined. In the testes of newborn rats, intratesticular blood vessels are poorly developed, fragile to pressure, highly permeable to dyes and not organized in their relation to interstitial cells or seminiferous tubules (Kormano, 1967a, b). Within the first 15 days of postnatal life, little organization of the blood vessels takes place as indicated by the result of a microangiographic study (Kormano, 1967a). The organization into the mature capillary network, consisting of intertubular capillaries and peritubular capillaries, develops between the ages of 20 and 35 days (Kornamo, 1967a). Another study examined the ultrastructural aspects of the developing testicular microvasculature in immature golden hamsters and compared with that in adult hamsters (Mayerhofer & Bartke, 1990). Angiogenic process, as indicated by the presence of migrating endothelial cells, is most conspicuous between days 8 and 25 of postnatal development, almost absent on days 30 and 35 and not detected in the testes of 3-month-old hamsters. This study also indicates that the formation of intertubular capillaries precedes the development of peritubular vessels. This general sequential pattern in the development of the testicular microvessels is in accordance with results of a previous microangiographic study on the postnatal formation of testicular microvasculature in the rat testis (Kornamo, 1967a). In another study, the population of endothelial cells in mouse and rat testes appears to develop in a similar fashion except for the fact that the major increase in the number per testis occurs somewhat earlier in mice than in rats (day 18 and day 21, respectively) (Hardy, Zirkin & Ewing, 1989). The endothelial cells show a more or less constant proliferative activity after birth (Vergouwen *et al.*, 1991) at a level which is somewhat higher than that of the

other interstitial cells. The level of proliferative activity is probably enough to account for the observed increase in the number of endothelial cells and the growth of new blood vessels within the testis.

1.7 Aims of the present study

The aims of the present study are to use endothelial cell proliferation as an index of angiogenesis (a) to examine whether this parameter is stimulated in adult rat testes following the exposure to a single dose of hCG, and how this may be related to the presence or the activity of Leydig cells and testicular macrophages, and the levels of spermatogenesis in the seminiferous tubules, and (b) to examine how the magnitude of this parameter will change during the postnatal development of the rat testes. In some experiments, the changes in the number of proliferating endothelial cells will be related to the blood vessel density within the testis. In addition, testis weight and their serum testosterone concentration will also be measured in the animals used in these studies.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats were used and they were obtained from the Animal House, The Chinese University of Hong Kong. They were maintained under a controlled environment of 21 ± 2°C, humidity below 75%, and a 12 h light (06:00h - 18:00h) / 12 h dark (18:00h - 06:00h) cycle. The animals had free access to rat chow and tap water.

Adult rats used in this study were about 90 days old and had a body weight range of 400-450g. In the age study, animals of specific ages were used and the day of birth was taken as the age of day 1.

2.2 Experimental designs

2.2.1 Testicular angiogenesis in adult rats - hormonal stimulation by hCG

2.2.1.1 Changes with time after hCG treatment

In the treatment groups, each animal received a single subcutaneous injection of 100 IU hCG (Pregnyl, Organon, Netherland) in 0.2 ml phosphate-buffered saline (PBS) containing 0.01% bovine serum albumin (BSA) (Fraction V, protease-free; Sigma Chemicals Co., St. Louis, MO, USA) as the carrier protein. The timing of hCG injection was such that the animals were killed on the same day at various times ranging from 12 hours to 7 days post-hCG injection (Figure 2.1). The control group was injected with 0.2 ml vehicle (saline containing 0.01% BSA) 24 hours before being killed. All animals were killed by decapitation and trunk blood was collected for the measurement of serum testosterone concentration by radioimmunoassay. Both testes were collected and weighed before one was perfused with fixative (i.e. Bouin solution) and the other with Indian ink (Staedtler, Nurnberg, Germany) for the

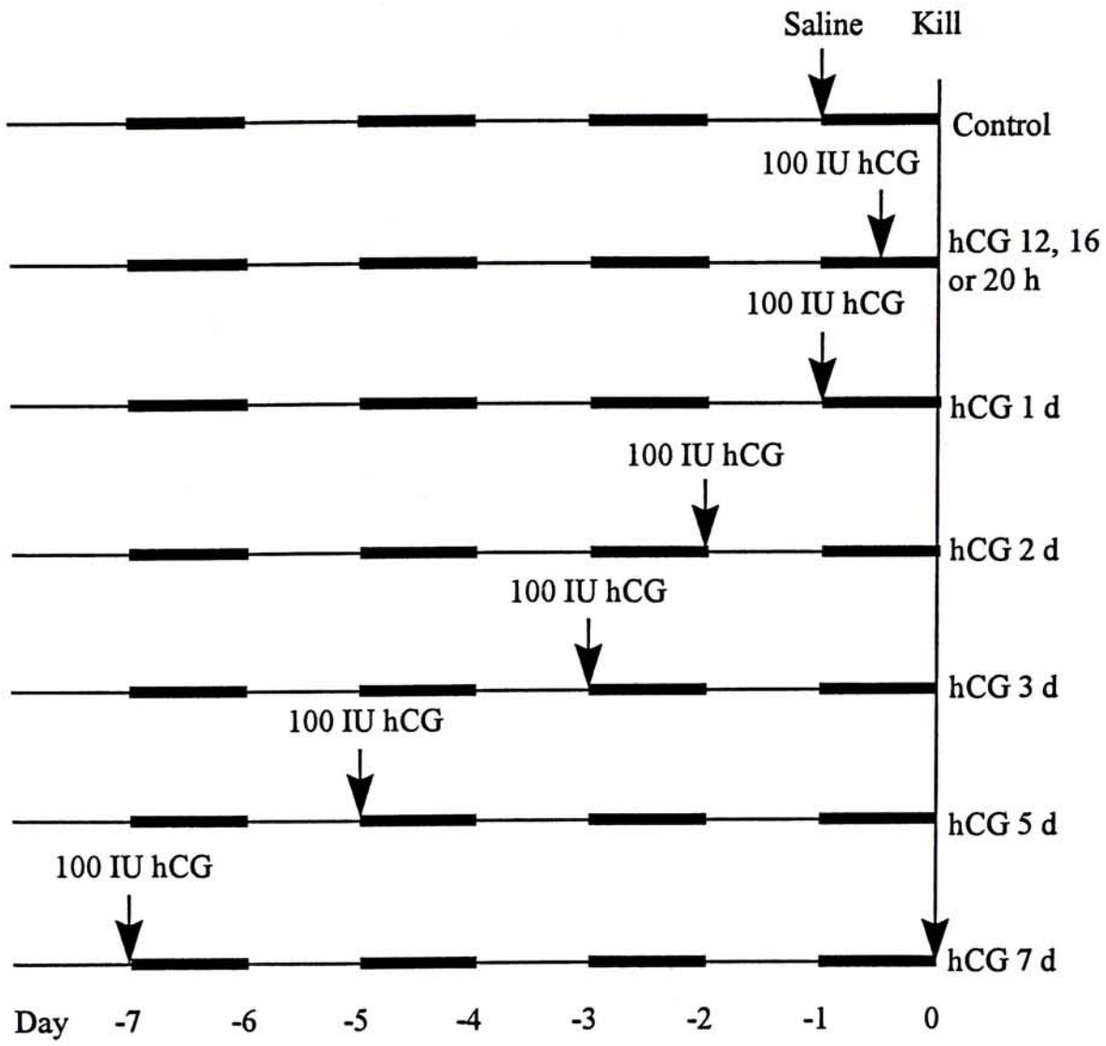

Figure 2.1 Experimental design used in the study of the effect of a single subcutaneous injection of 100 IU hCG on endothelial cell proliferation in adult rat testes. The animals were examined over a period ranging from 12 hours to 7 days post-hCG.

quantification of proliferating endothelial cells and blood vessel density, respectively. Other details on the fixation and processing of the testes are described later.

2.2.1.2 Effect of Leydig cell depletion

In order to investigate whether Leydig cells are involved in mediating the angiogenic response of the testis to hCG, ethane-1,2-dimethane sulphonate (EDS; synthesized by the Chemistry Department, The Chinese University of Hong Kong) treatment was used to selectively destroy the Leydig cells (Jackson & Jackson, 1984). EDS was dissolved in a dimethylsulphoxide (DMSO):water mixture (1:3 v/v) to a concentration of 75 mg/ml. Rats were injected intraperitoneally with a single dose of EDS (75 mg/kg bw) or the vehicle (DMSO:water) 3 days prior to receiving a single subcutaneous injection of 100 IU hCG (or saline or the treatment control) and studied at 1 and 2 days post-hCG. Details of the experimental design are shown in Figure 2.2. At the time when the rats were killed by decapitation, trunk blood was collected for the measurement of serum testosterone concentration by radioimmunoassay, and testes were removed and processed for immunohistochemical localization of proliferating endothelial cells (details given later).

2.2.1.3 Effect of Leydig cell suppression by subcutaneous testosterone-filled silastic implants

Rats received subcutaneous testosterone-filled silastic implants (Dow Corning, 602-305; i.d. 1.98 mm, o.d. 3.18 mm) of either 3 cm or 25 cm in length for eight weeks to achieve Leydig cell suppression. The two lengths of testosterone implants were chosen based on the fact that the 3 cm implant would produce normal levels of serum testosterone (Robaire et al., 1979) but could not provide adequate hormonal

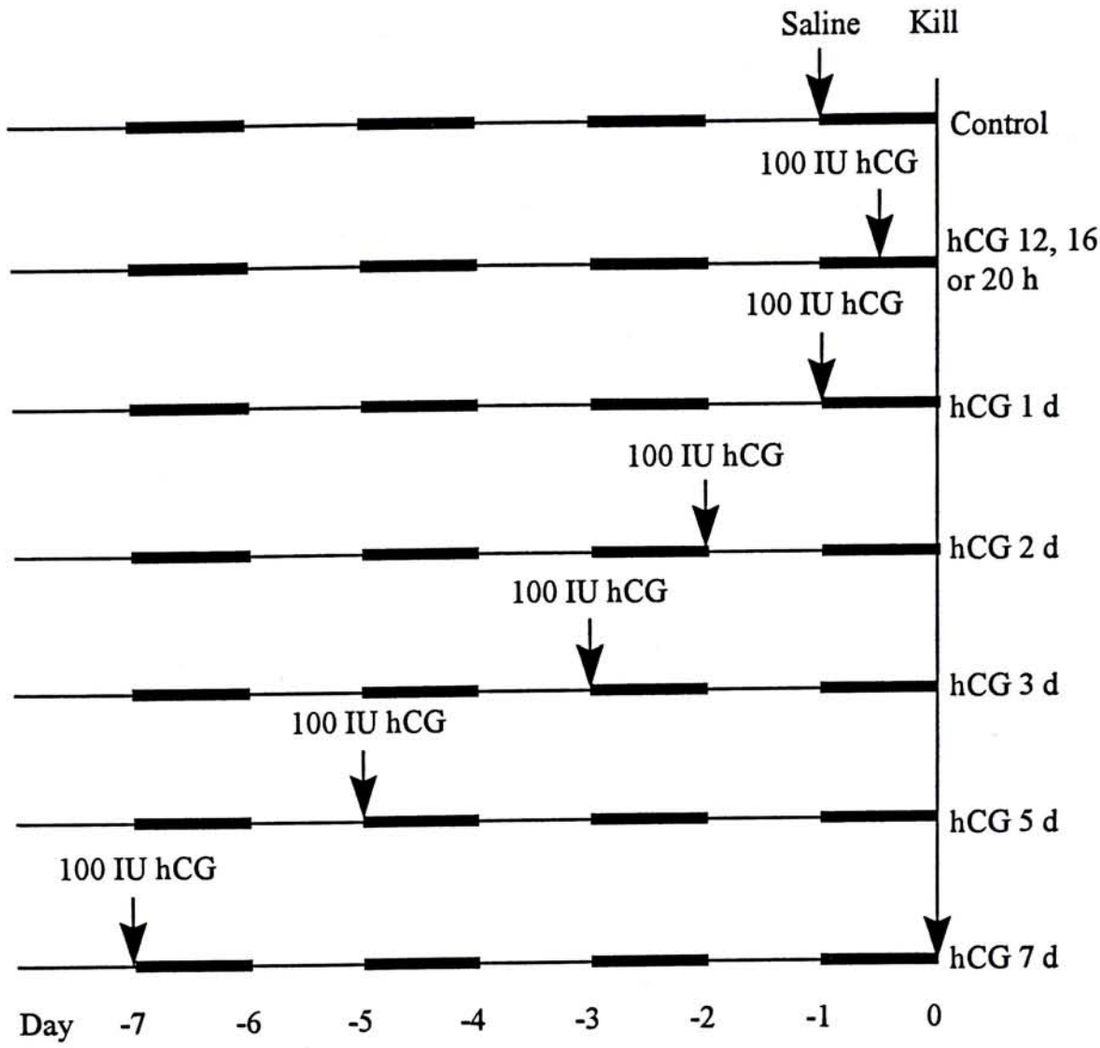

Figure 2.1 Experimental design used in the study of the effect of a single subcutaneous injection of 100 IU hCG on endothelial cell proliferation in adult rat testes. The animals were examined over a period ranging from 12 hours to 7 days post-hCG.

support to maintain normal spermatogenesis, while the 25 cm implant would maintain near normal levels of spermatogenesis but in the presence of supraphysiological levels of circulating testosterone (Sun et al., 1989; McLachlan et al., 1994). Age-matched animals without bearing any subcutaneous silastic implants were used as the control. After 8 weeks of treatment, half the number of animals in the control and treatment groups received a single subcutaneous injection of 100 IU hCG while the other halves were injected with saline. They were killed 2 days post-hCG (or saline) for the quantification of proliferating endothelial cells in the testes and for the measurement of serum testosterone concentration. Details of the experimental design are shown in Figure 2.3.

2.2.1.4 Effect of testicular macrophage activation

Testicular macrophages were activated by intratesticular injection of polystyrene latex beads according to the procedure described by Kerr & Sharpe (1989). Under light ether anaesthesia, rats were given a unilateral intratesticular injection of 2% polystyrene latex beads (0.8 μm diameter, Sigma) in 150 μl sterile saline into the central part of the right testes, and 150 μl sterile physiological saline into the contralateral testes. Three days later, half of the animals were injected subcutaneously with 100 IU hCG while the rest were injected with saline and served as the corresponding control. The animals were studied at 1- and 2-day post-hCG. Sera were collected for the measurement of testosterone concentration and testes were fixed for histological examination and quantification of proliferating endothelial cells. Details of the experimental design are summarized in Figure 2.4.

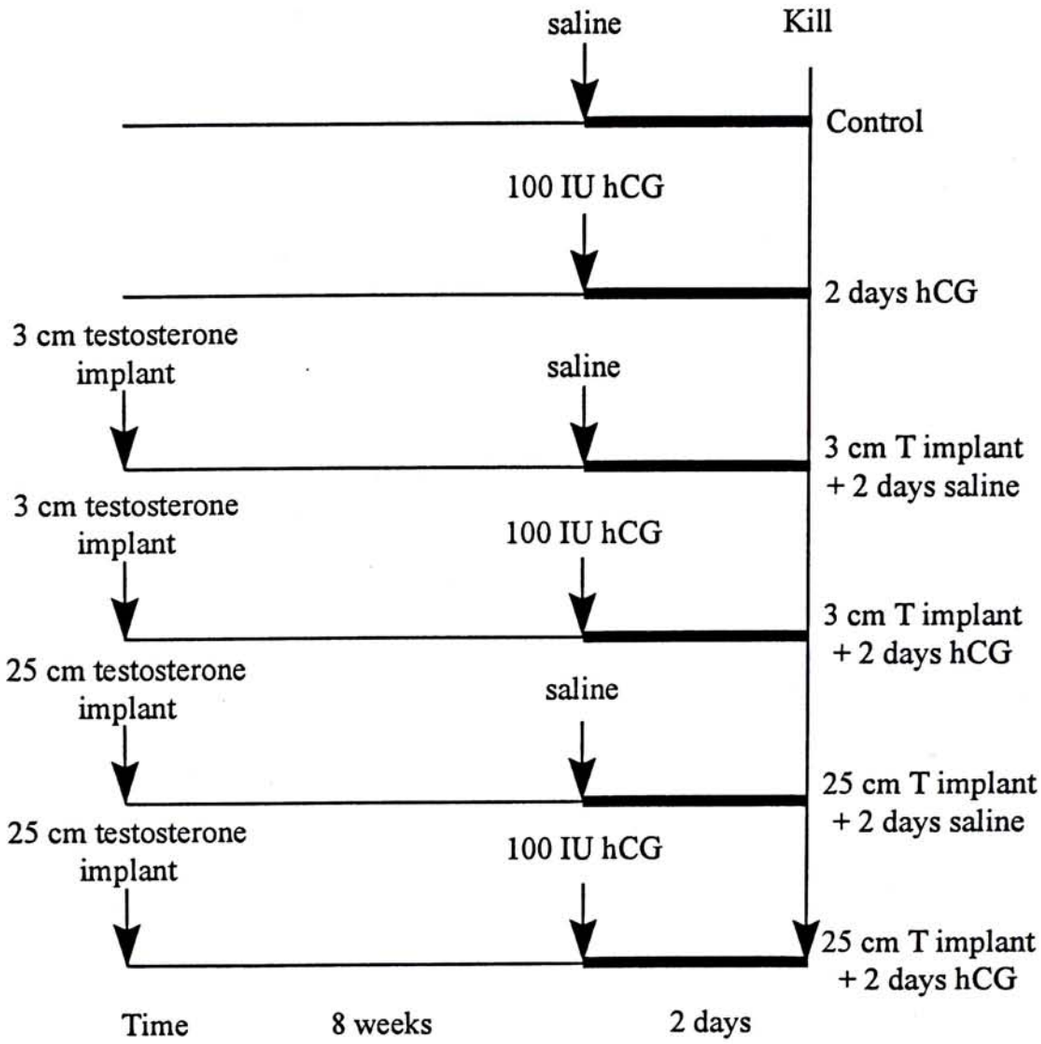

Figure 2.3 Experimental design used in the study of the effect of Leydig cell suppression by 3 cm or 25 cm testosterone (T) - filled subcutaneous silastic implants on hCG-induced endothelial cell proliferation in adult rats testes. The animals were examined at 2 days post-hCG.

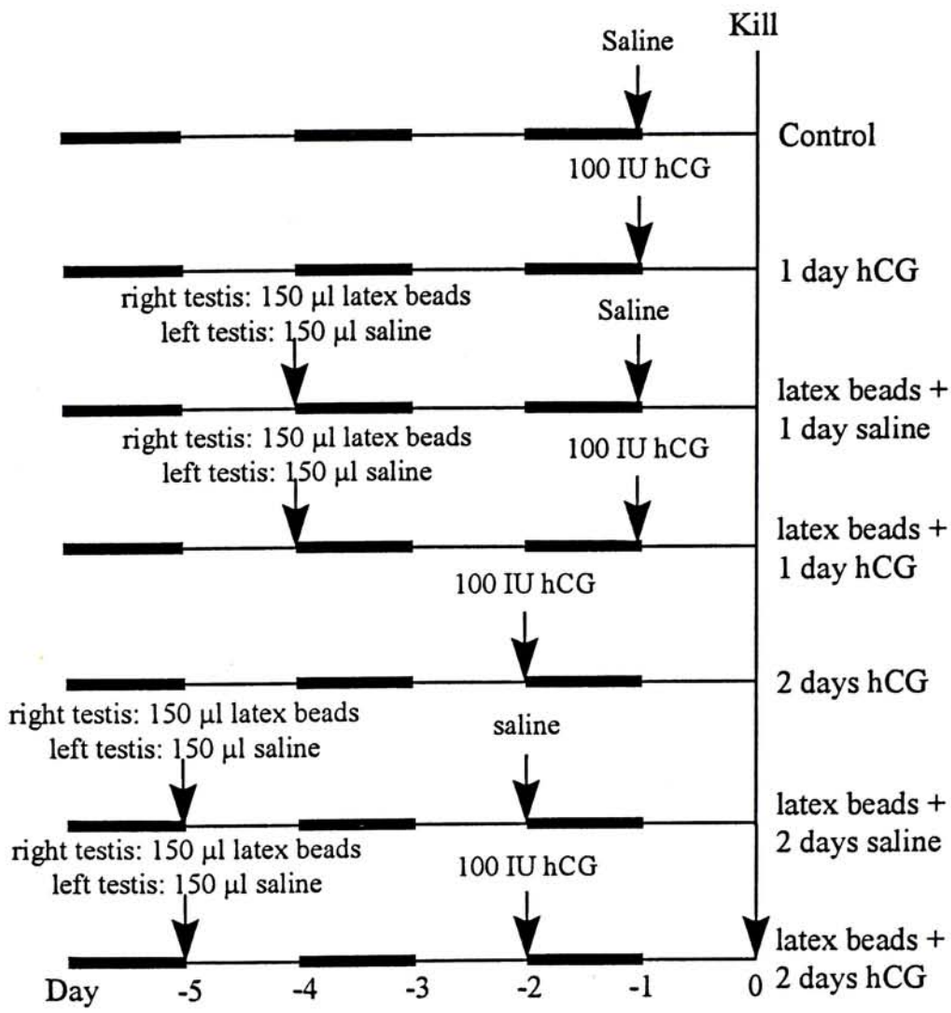

Figure 2.4 Experimental design used in the study of the effect of testicular macrophage activation by polystyrene latex beads on hCG-induced endothelial cell proliferation in adult rat testes. The animals were examined at 1 day and 2 days post-hCG.

2.2.1.5 Effect of testicular macrophage depletion

Testicular macrophage depletion was achieved by intratesticular injection of liposome-entrapped dichloromethylene diphosphate (Cl_2MDP-lp) according to the method described by Bergh et al. (1993a & b). Cl_2MDP was a gift from Boehringer-Mannheim GmbH (Germany) and the preparation of Cl_2MDP-lp was described in a later section. Under light ether anaesthesia, the animals received bilateral intratesticular injection of 150 µl Cl_2MDP-lp per testis for the treatment group or 150 µl liposome encapsulated phosphate buffered-saline (PBS-lp) per testis for the control group using a 27 gauge hypodermic needle. The injection was given through the scrotal skin and the scrotal sac (i.e. tunica vaginalis) without exposing the testes. After identifying the outline of the testis underneath the scrotal skin, the tip of the needle was inserted into the central part of the testis and the injection was carried out slowly over a period of 20 seconds. Two weeks after Cl_2MDP-lp treatment (or sham injection) when in the previous report, the numbers of testicular macrophages were found to be at its lowest (Bergh, Damber & van Rooijen, 1993a), half of each group was injected subcutaneously with 100 IU hCG while the remaining half was injected with saline. On the same day, all animals received intraperitoneal injection of 3% trypan blue in saline (0.75 ml/kg bw) to label the testicular macrophages (Niemi, Sharpe & Brown, 1986), if any, that were present. Two days post-hCG, the rats were killed by decapitation for the collection of sera and testes. Details of the experimental design are shown in Figure 2.5.

2.2.2 Developmental changes in testicular angiogenesis

Rats of specific ages (10, 20, 30, 40, 50, 60 and 70 days old) were used and they were killed by decapitation. Trunk blood was collected for the measurement of

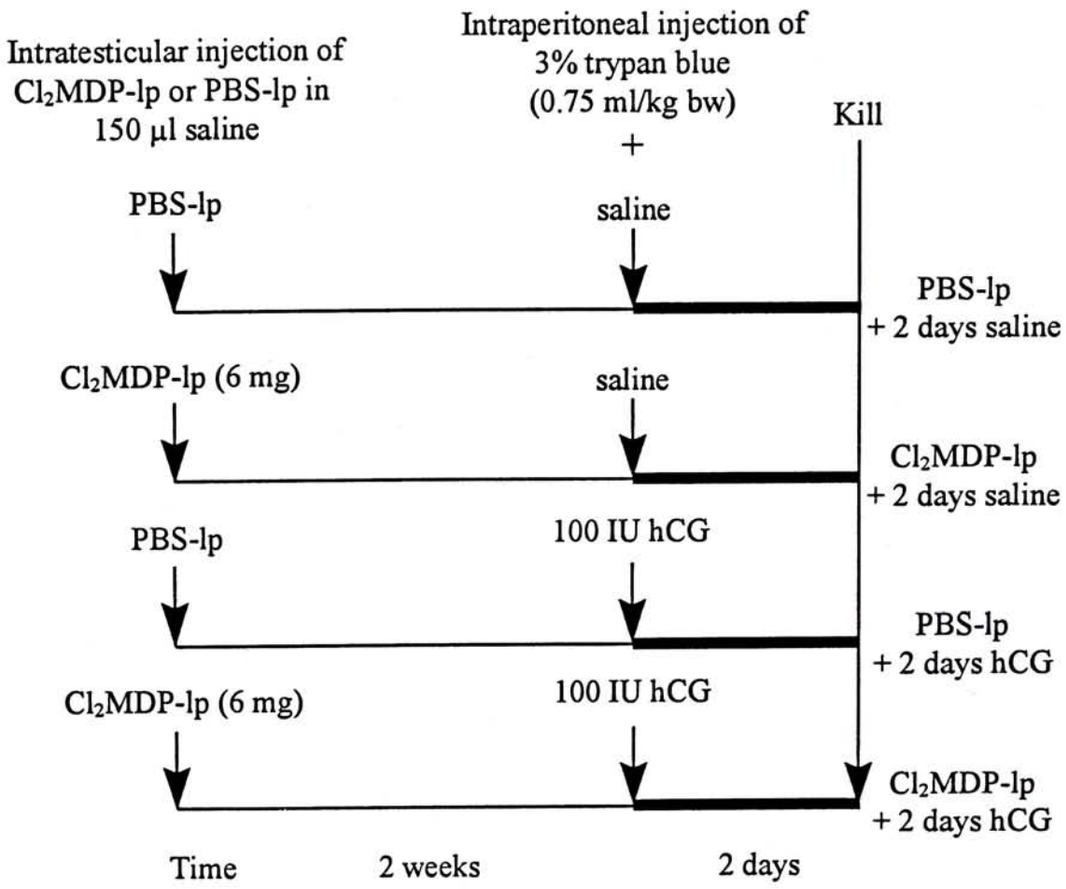

Figure 2.5 Experimental design used in the study of the effect of testicular macrophage depletion by liposome-entrapped dichloromethylene diphosphonate (Cl_2MDP-lp) treatment on hCG-induced endothelial cell proliferation in adult rat testes. The animals were examined at 2 days post-hCG.

serum testosterone. Testes were perfused with Bouin solution or Indian ink before being processed for immunohistochemical staining of proliferating endothelial cells or quantification of blood vessel density, respectively. In younger animals (10-30 days old), the descending aorta was cannulated for lower body perfusion to fix the testes or to infuse with Indian ink. In older animals, the testes were excised and weighed before being perfused through the testicular subcapsular artery. The data obtained from this study were examined together with values obtained from normal adult rats of about 90 days of age.

2.3 Perfusion of testes with fixative or Indian ink

In adult and 40-70 days old rats, testes were excised (without the epididymides) and weighed after the animals were killed by decapitation. Using a scapel blade (size #24, Swann-Morton, Sheffield, England), a small cut was made in the testicular subcapsular artery close to where it enters the tunica albuginea at the cranial pole of the testis and along its course down the epididymal margin of the testis. A cannula constructed from a polythene tubing (0.5 mm i.d., 1.0 mm o.d., 800/110/160, Portex, UK) and heat-drawn to a tip diameter of about 100 μm diameter was inserted into the subcapsular artery along the direction of normal flow. One to two millilitres of pre-warmed (25-30°C) heparinized saline (50 U/ml in normal saline; sodium salt of heparin from porcine intestinal mucosa; Sigma, USA) were then infused through the cannula into the testis by hand using a 5 ml syringe fitted with a gauge 23 needle (Terumo, Tokyo, Japan), until all the blood was flushed out. The saline infusion was then followed by 1.5-2.0 ml of Bouin fixative or Indian ink depending on whether the tissue was subsequently used for the quantification of proliferating endothelial cells or blood vessel density, respectively. In the infusion,

care was taken to prevent the entry of any air bubbles, and the volume of infusate was adjusted until there were a complete removal of blood and a uniform distribution of the fixative/ink. The perfused testes were then immersion-fixed in Bouin solution for 24 hours at room temperature, followed by another 24 hours at 4°C. After the first 24 hours, the testes were cut using microtome blades (S35, Feather, Japan) into 2 mm thick slices at right angle to the cranial-caudal axis.

For 10 to 30 days old rats, testes were perfused *in situ* after cannulating the descending aorta. Under ether anaesthesia, the thoracic cavity of the animal was cut open to expose the descending aorta behind the right lung. A cannula constructed from a polythene tubing (0.5 mm i.d., 1.0 mm o.d., 800/110/160, Portex, UK) was inserted into the aorta through a small incision and tied in place using surgical sutures (4.0, Pearsalls, England). Pre-warmed heparinized saline (25-30°C) was infused using a syringe pump fitted with a 5 ml syringe at a rate of 0.8 ml per minute. After the blood was removed, Bouin fixative or Indian ink (about 2 ml per animal) was infused at the same rate. After perfusion, the testes were removed and immersion-fixed in Bouin solution for 2 days as described above. The testes were bisected after 24 hours at right angle to the cranial-caudal axis.

2.4 Processing of the testes for histological sections

After fixation, the testes were transferred to 70% alcohol and washed by several changes to remove the picric acid left in the tissue from the Bouin fixative. In the subsequent steps of tissue processing, a slice of tissue (about 2 mm thick) was taken from the central part of each testis and put through a tissue processor (Department of Anatomy, The Chinese University of Hong Kong) for dehydration in alcohol, clearing in xylene and embedding in paraplast (Paraplast X-TRA, melting

point 50-54°C, Oxford Labware, St. Louis, MO, USA). The timing of the above steps differed depending on the size of the testis (i.e. the age of the rats from which the testes were obtained) and it ranged from 30 min to 1 hour with 3 changes of absolute ethanol and 2 changes of xylene. Serial sections (5μm thick) were prepared using a microtome (Model 1130, Biocut, Reichert-Jung, Germany) and every tenth section was mounted on a glass slide coated with 1% 3-aminopropyltriethoxysilane (APES) (Sigma) in ethanol (Maddox & Jenkins, 1987).

2.5 Immunohistochemical staining for proliferating cell nuclear antigen (PCNA)

To examine the changes in the levels of testicular angiogenesis, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was used as a marker to identify the number of proliferating endothelial cells, which in turn was taken as an index of angiogenesis.

Reagents used for immunohistochemical staining were from Zymed Laboratories Inc. (South San Francisco, USA) and they are based on the streptavidin-biotin-peroxidase system. The procedure was carried out according to manufacturer' s instructions with some modifications. Paraplast-embedded 5 μm testis sections were dewaxed in (R)-(+)-limonen (Merck, Germany) and rehydrated through a down series of ethanol and finally equilibrated in 10 mM phosphate-buffered saline (PBS, pH 7.4). The same buffer was used in subsequent washes (3 times, 2 min each) between steps and in the dilution of antibodies and reagents except the chromogen. The tissue endogenous peroxidase activity was quenched by treatment with 3% H_2O_2 (Merck, Germany) in PBS for 10 min. Sections were then incubated overnight at 37°C with a mouse monoclonol antibody against PCNA (Clone PC10, IgG2a subclass, Dako, Denmark) at a 1:100 dilution in PBS containing 10%

foetal calf serum (Gibco, MD, USA) to block non-specific binding. This was followed by 10 min incubation with a biotinylinated goat anti-mouse IgG (H+L) second antibody at a dilution of 8 μg/ml. The second antibody binding was subsequently localized by incubating the sections for 10 min with horseradish peroxidase-conjugated streptavidin at 1:400 dilution. The peroxidase label was then visualized by developing the sections for 15-30 min in acetate buffer containing 3-amino-9-ethylcarbazole (AEC) in N,N-dimethylformamide and 0.03% H_2O_2. After thorough rinsing with distilled water, the sections were mounted in an aqueous mounting medium - polyvinyl pyrrolidone (Bancrofts & Stevens, 1996) without counterstaining with haematoxylin.

In validating this method for the immunostaining of PCNA in testis sections, both the omission of the primary antibody and its replacement by pre-immune mouse immunoglobulin of the same isotype (mouse IgG2a, Clone GC270, Serotec, UK) had been tested as the negative controls. No non-specific or background staining could be detected.

2.6 Immunohistochemical staining for vascular endothelial growth factor

In studying the effect of Leydig cell suppression on hCG-induced endothelial cell proliferation in adult rat testes, the levels of immunoreactivity of an angiogenic factor - vascular endothelial growth factor (VEGF), localized in Leydig cells and Sertoli cells of the rat testis (Au et al., 1997) were examined by immunohistochemical staining. The methods were the same as those earlier described for PCNA with some minor modifications. After blocking the endogenous peroxidase activity, the testis sections were subjected to an antigen retrieval procedure in which the slides were boiled in 0.01 M citrate buffer (pH 4.1) for 20 min at 95°C and then left to cool in the

same buffer for 20 min at room temperature. The VEGF antibody used in this study was commercially available (Santa Cruz Biotechnology, Santa Cruz, CA, USA). It is an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide which corresponds to amino acids 1-20 at the amino terminus of VEGF. This region of VEGF is common among the different isoforms. Previous studies had reported the use of this antibody in the immunhistochemical staining of VEGF in rat tissues (Collin & Bergh, 1996). The VEGF antibody was used at 1:200 dilution and incubated with the section for 1 hour at room temperature. The second antibody was a biotinylated goat-anti-rabbit IgG (H+L) used at a dilution of 8 μg/ml. After immunostaining, the sections were counterstained with haematoxylin.

2.7 Quantification of PCNA-positive endothelial cells

Proliferating cells were identified based on the positive nuclear staining for PCNA. Since within the intertubular areas of the testis, there were other cell types which could divide and hence stained positive for PCNA (e.g. Leydig cells, macrophages, vascular smooth muscle cells, pericytes), the endothelial cells were distinguished according to the criteria described by Hardy *et. al.* (1989). Endothelial cells are fusiform cells with cytoplasmic extensions that form the walls of the blood vessels. Although these cells are in intimate contact with pericytes, the two can be distinguished because pericytes are located outside of the vessel, and as well their nuclei typically bulge away from the lumen of the vessel. In contrast, the nucleus of the endothelial cell bulges inward and deflects the circular contour of the vessel lumen.

The number of PCNA-positive endothelial cell was determined by counting the entire testis section at 400x magnification. This was necessary since under normal

condition in adult testes, the number of proliferating endothelial cells is extremely low and random sampling was found to create large errors. At least five sections (taken every ten sections apart) were counted from each testis. The number of sections to be sampled was established based on a pilot analysis of the sampling variances which had to be less than 5%.

In most studies, the number of proliferating endothelial cell was normalized against the tissue section area (number/mm^2). However, in the developmental study and those experimental treatments where the ratio of tubular and intertubular area in the testis might change, the number of proliferating endothelial cells was also expressed as per unit intertubular area.

2.8 Quantification of blood vessel density

Blood vessel density was quantified using a colour image analysis software (Video Pro 32, Leading Edge Pty. Ltd., Adelaide, Australia; installed on a 486-33MHz PC computer) on serial sections obtained from ink-perfused testes. The sections were dewaxed and mounted in a permanent mountant (DPX mountant, BDH, England) without staining. For each testis section, 20 randomly selected fields were captured at 100x magnification through a light microscope (Optiphot-2, Nikon, Japan) fitted with a video camera (Model TK-1280E, JVC, Japan) that input the signal into a PC computer (486-33MHz) and displayed the image (640 pixels wide by 442 pixels high) on a computer monitor (15", Sony Trinitron, Model CPD-15SF2, Japan). Using the threshold and measure function of the image analysis software, the ink-filled features indicating the blood vessels were discriminated against the unstained background and their numbers were counted (Figure 2.6). The actual area occupied by each displayed field on the computer monitor was determined using a

stage micrometer. From the total area occupied by the number of fields (total = 20) that were being sampled, the blood vessel density per unit section area was determined.

2.9 Estimation of intertubular area in testis sections

The percentage of the whole section area occupied by the intertubular area was estimated in those slides used for the quantification of PCNA-positive endothelial cells. Using the colour image analysis software (Video Pro 32) and under 400x (for 10-day old rats) or 200x (for other age groups and studies using adult animals) magnification, the boundaries between the tubular and intertubular area on the testis section were traced out on the computer monitor using a pointing device (i.e. PC mouse) and the edit/draw function of the software. The intertubular regions were then discriminated using the edit/fill function and their areas measured (Figure 2.7). For each testis section, 20 randomly selected fields were sampled. Care was taken to avoid those regions where there was an artifactual shrinkage/separation of tubular and intertubular tissues caused by the histological processing. From the percentage of total area occupied by intertubular tissue and the actual total area that was being sampled, the intertubular area present in each testis section was determined and used in the expression of the PCNA-positive endothelial cell count.

2.10 Preparation of liposome-entrapped dichloromethylene diphosphonate (Cl_2MDP-lp)

The liposome-entrapped dichloromethylene diphosphonate (Cl_2MDP) was prepared according to published methods (van Rooijen, 1989) with slight modifications. 75 mg phosphatidylcholine (Sigma) and 11 mg cholesterol (Sigma)

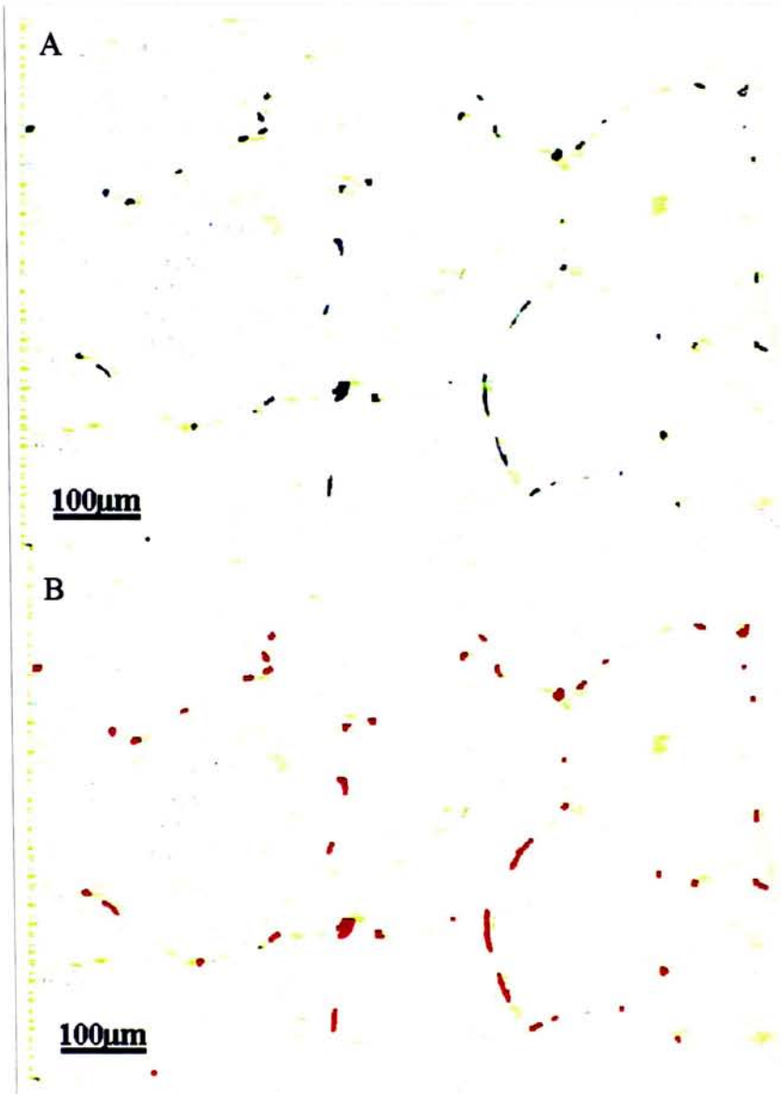

Figure 2.6 Use of the colour image analysis system to measure the density of blood vessels per unit area in testis sections. In (A), the blood vessels filled with ink were seen as numerous black features against the pale background of the unstained section. In (B), it is an identical field as shown in (A) with the ink-filled blood vessels being discriminated by the image analysis software and covered by red overlays. When setting the threshold in the above step, care was taken to avoid separate features being joined together or *vice versa*.

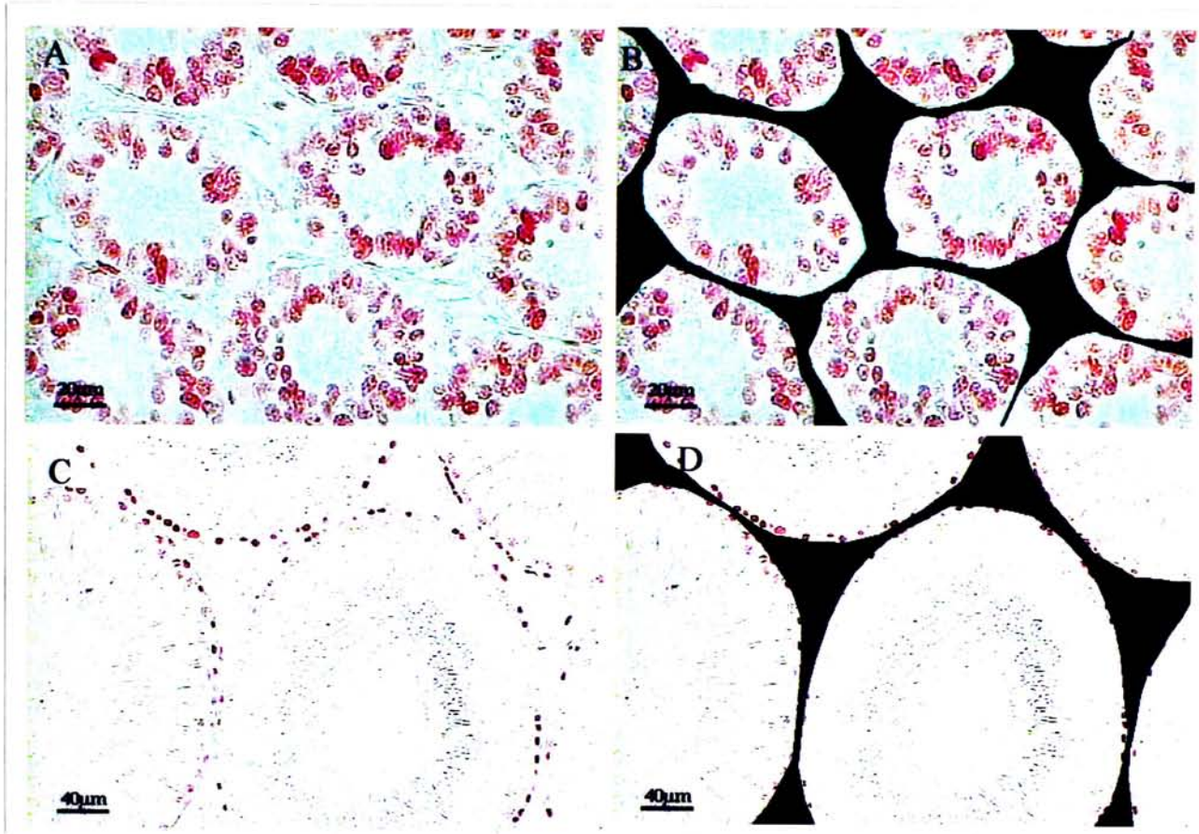

Figure 2.7 Use of the colour image analysis system to determine the intertubular area in testis section. Testis sections of 10 days old rats (A & B) and adult rats (C & D) were shown to have their intertubular areas measured by a black overlay (B & D) generated by the image analysis software after the boundaries between tubular and intertubular areas were traced out manually using a point device. The percentage of the total testis section area occupied by intertubular tissues appears to decrease with increasing age of the rats. The red colour associated with the nuclei of some cells represents the positive immunostaining for PCNA.

were dissolved in 20 ml methanol/chloroform (1:1, v/v) (Merck) in a 250 ml round bottom flask. The organic phase formed was removed at room temperature under a stream of nitrogen using a rotary evaporator (Labconco, Kansas, USA). The lipid film remaining was reconstituted in 10 ml chloroform which was similarly subjected to rotary evaporation at room temperature under a stream of nitrogen. At the end, the thin film that coated the bottom of the flask was dispersed either in 10 ml PBS containing 160 mg Cl_2MDP for preparing Cl_2MDP-lp, or in 10 ml PBS alone for preparing PBS-lp. After completely removing the lipid film, the suspension was left standing for 2 hours to determine whether it remained milky, indicating successful formation of the liposomes. The suspension was then subjected to 3 min sonication at room temperature in a waterbath sonicator (Branson, Shelton, Conn., USA). The liposomes were given another 2 hours to swell before they were spun down (100,000 g for 30 min at 16°C) and washed twice in PBS. Finally, the liposomes were resuspended in 4 ml PBS and 150 µl of this suspension were used for intratesticular injection.

2.11 Radioimmunoassay of serum testosterone

Sera collected were aliquoted and stored at below -20°C until being assayed for testosterone. For each animal, 100-300 µl of serum were used and placed in a glass tube (150x16 mm, Corning, N.Y., USA). Testosterone was extracted by vortexing each serum sample with 4 ml diethyl ether (Merck GR grade) for 1 min. The aqueous layer was allowed to settle for 30 min and then snap-frozen in liquid nitrogen. The ether layer was decanted into another glass tube (75x10 mm, Corning) and evaporated under a stream of air in a 40°C water bath for 15-20 min. The dried residue was reconstituted in assay buffer to the appropriate dilution and allowed to

stand at room temperature for 1 hour. Recovery of testosterone extraction was determined with the use of 100 µl working tracer diluted 1:3 in assay buffer. Following extraction, it was reconstituted in 300 µl assay buffer and duplicates of 100 µl were counted and compared with a working tracer of 1:9 dilution. In the present studies, no corrections were applied to the assays since the estimated percentages of recovery were all above 90%.

The testosterone antibody was developed in-house by Drs. C.C. Wong & C.L. Au (Department of Physiology, The Chinese University of Hong Kong). It was raised in rabbits against testosterone-3-oxime-BSA conjugate (Sigma) and used at an initial dilution of 1:15,000. Under the assay conditions used, it showed 100% cross-reactivity with dihydrotestosterone. The tracer [(1,2,6,7-H^3)-testosterone, 98.0Ci/mmol] was purchased from Amersham (Buckinghamshire, England). An appropriate volume of the stock solution in toluene:ethanol (9:1) was evaporated to dryness and then reconstituted in assay buffer to 20,000 cpm/100 µl/tube. Anhydrous testosterone was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as the standard covering a range of 25-1600 fmol/100 µl/tube. At the end of the assay, the separation of free from antibody bound testosterone was achieved by incubating each tube with 400 µl/tube of dextran-coated charcoal [0.0625 g dextran T-70 (Pharmacia, Sweden) and 0.625 Norit-A charcoal (Serva, Texas, USA) in 100 ml assay buffer] at 4°C for 30 min. The dextran-charcoal was subsequently separated by centrifugation at 1,000 g for 15 min. The supernatant which contained the antibody bound radioactivity was decanted into scintillation vials, equilibrated with the scintillation cocktail [0.4 g POPOP, 4 g POP in 700 ml toluene and 300 ml triton X-100] for 1 hour and counted in a liquid scintillination counter (Beckman LS-6000,

USA). The testosterone concentrations in the samples were computed from the standard curve using a programme prepared by the Radioimmunoassay Unit, Department of Medicine, University of Hong Kong.

The sensitivity of the testosterone radioimmunoassay was avearged to be 20.6±7.5 fmol/tube (mean±SD) (n=8). The intra-assay and inter-assay coefficient of variation were 6.7% and 12.7%, respectively (n=12). All serum samples from the one experiment were measured in the same essay.

2.12 Statistical analyses

Data were expressed as mean±SE. Statistical analyses were performed using a commercially available software package (SigmaStat for Windows Version 1.0, Jandel Scientific Software, San Rafael, CA, USA). Parametric tests were used wherever possible, unless the data failed in the normality test. For multiple comparisons among many groups, one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was used, and for multiple comparisons versus a single control group, one-way ANOVA followed by Dunnett' s test was used. For multiple comparisons among many groups using a non-parametric test, Kruskal-Wallis one-way ANOVA on ranks was used together with the Dunn's test.

3. Results

3.1 hCG-induced increase in endothelial cell proliferation in adult rat testes

3.1.1 Testicular histology

The PCNA-positive endothelial cells were identified based on the specific nuclear staining for PCNA in those cells lining the wall of blood vessels and with their nuclei protruding towards the lumen (Figure 3.1). Since the testes were fixed by vascular perfusion, the blood vessels could be easily distinguished from the lymphatic vessels by their dilated lumens which were clear of plasma and blood cells.

In normal adult rat testes, there were very few PCNA-positive endothelial cells (Figure 3.1) and they were found in blood vessels throughout the entire testis section. After adult rats were given a single subcutaneous injection of 100 IU hCG, there was a marked increase in the number of PCNA-positive endothelial cells suggesting a hCG-induced proliferation of this cell type (Figure 3.1 and 3.2). Most of the increase in PCNA-positive endothelial cells was found lining the wall of large venules (Figure 3.1) although it was also found elsewhere in capillaries and occasionally in arterioles.

3.1.2 Changes in the number of PCNA-positive endothelial cells

In normal adult rat testes, the number of PCNA-positive endothelial cells was relatively few. For a 5 μm section taken from the equatorial region of the testis, it gave a mean value of about 20. However, 2 days after hCG stimulation, this number increased to a maximum of about 620/section. When comparisons were made based on the number of PCNA-positive endothelial cells per unit testis section area, an increase was evident 20 hours after hCG stimulation but it did not reach statistical significance ($P>0.05$) until 24 hours post-hCG (Figure 3.2). By 48 hours post-hCG, it reached a maximum value of about 30-fold above the control. After this, the

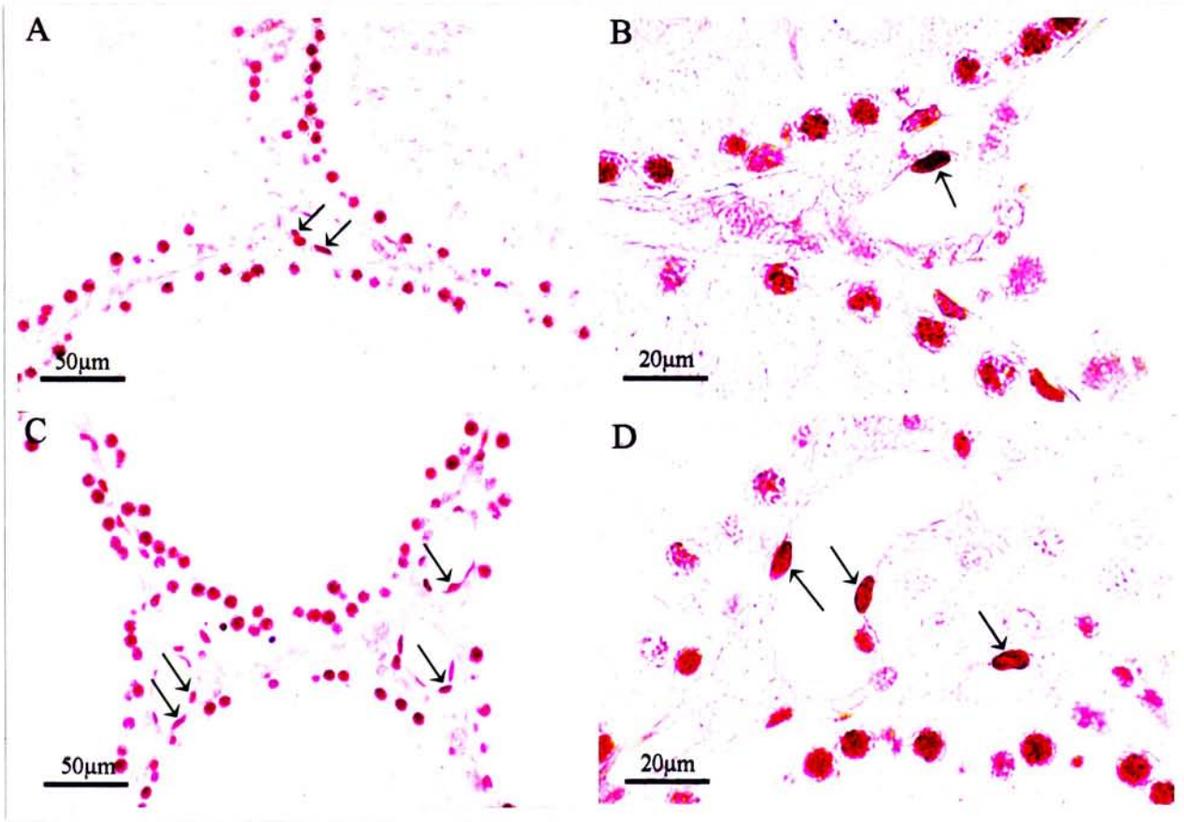

Figure 3.1 Representative proliferating cell nuclear antigen (PCNA) immunostaining in testes of adult rats that received a single subcutaneous injection of saline as in the control (A & B), or 100 IU hCG (C & D) 2 days prior to when the animals were killed for tissue collection. In both animals, the spermatogonia located at the base of the seminiferous tubules were stained positive for PCNA. However within the interstitium, PCNA-positive endothelial cells (indicated by arrows) were a lot more abundant in the testes of the hCG-injected animals than the control. Most of the PCNA-positive endothelial cells were found lining the wall of large venules or capillaries. In the control animals, PCNA-positive endothelial cells were a lot less abundant.

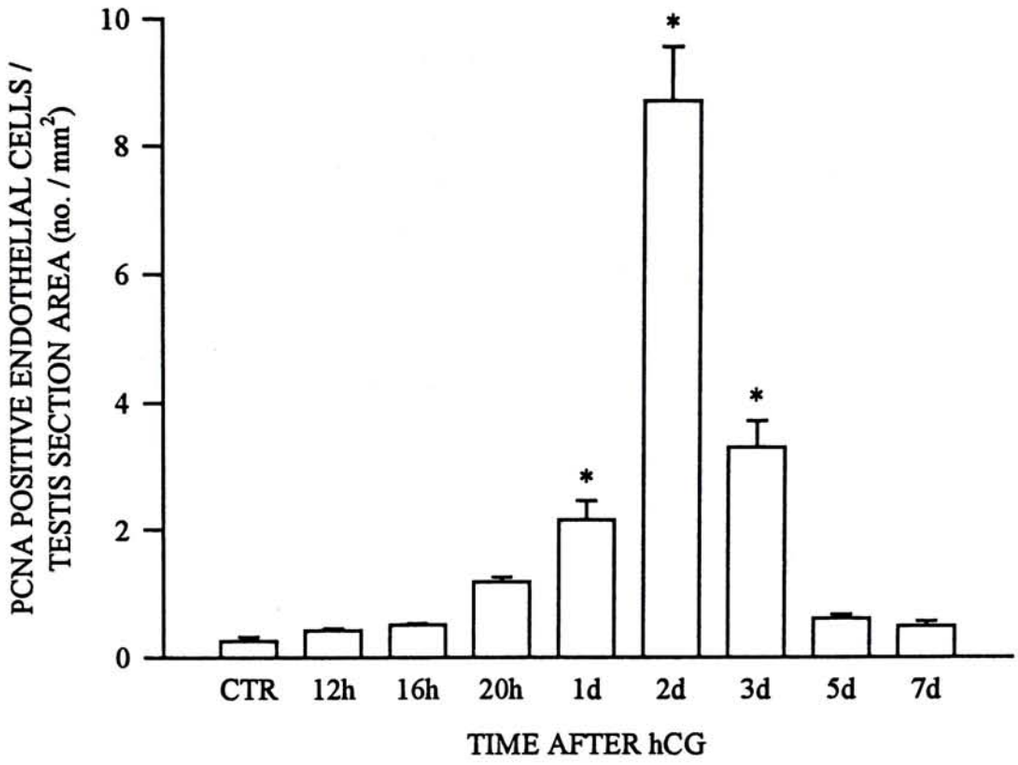

Figure 3.2 Changes in the number of PCNA-positive endothelial cells (per unit section area) in testes of adult rats at various times ranging from 12 hours to 7 days after receiving a single subcutaneous injection of 100 IU hCG.

Data represent mean±SE of 5-6 animals per group.
*P<0.05, compared with saline-injected control (CTR) using one-way ANOVA followed by Dunnett's test.

number of PCNA-positive endothelial cells gradually declined to reach a value insignificantly different from the control at 5 days post-hCG, and almost completely returned to the control value after 7 days (Figure 3.2).

3.1.3 Changes in blood vessel density

Associated with the hCG-induced increase in the number of PCNA-positive endothelial cells, the blood vessel density per unit testis section area exhibited a small and yet significant ($P<0.05$) increase at 3 and 5 days post-hCG (Figure 3.3). By 7 days after the hCG injection, it returned to a value insignificantly different from that of the control. The changes of this parameter including the first significant rise, the attainment of the maximum increase and the recovery back to normal values, all appeared to precede a similar pattern of changes in PCNA-positive endothelial cell number by about 1-2 days.

3.1.4 Changes in testis weight and serum testosterone concentration

The testis weight of the hCG-injected rats was significantly ($P<0.05$) elevated between 16 and 24 hours after treatment (Table 3.1). By 2 days post-hCG, it had already recovered back to a value insignificantly different from that of the control.

In line with previous reports, a single injection of hCG induced a biphasic serum testosterone response in adult rats (Figure 3.4). In the present study, the first peak of serum testosterone was noted at around 16-20 hours post-hCG. This was followed by a nadir at 2 days before a second peak appeared at 5 days post-hCG.

When the number of PCNA-positive endothelial cells per unit section area was correlated with the serum testosterone concentration based on their average values recorded at different times after the injection of hCG, a correlation coefficient (i.e. r

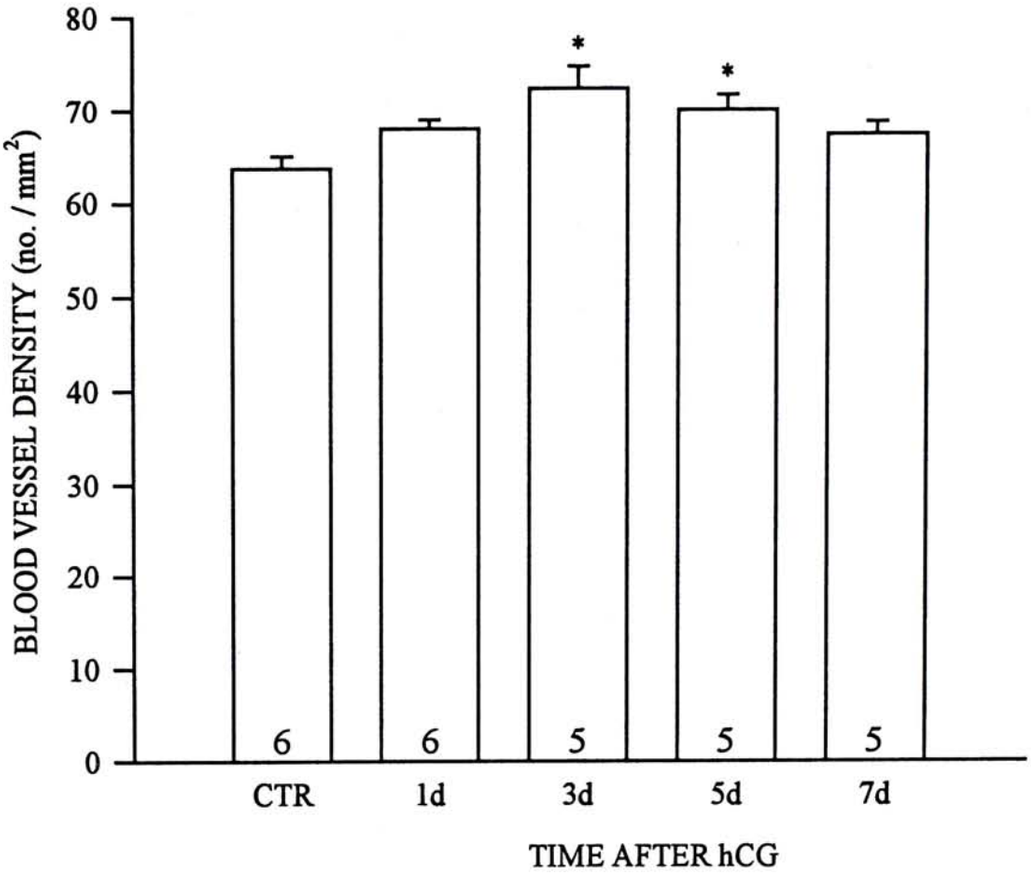

Figure 3.3 Changes in the blood vessel density in the testes of adult rats at various times after receiving a single subcutaneous injection of 100 IU hCG.

Data represent mean±SE with the number of animals given at the base of each bar. *P<0.05, compared with saline-injected control (CTR) using one-way ANOVA followed by Dunnett's test.

Time after hCG	Paired Testis Weight (gram)	No. of Animals
Control	3.416 ± 0.080	5
12 h	3.514 ± 0.112	6
16 h	3.853 ± 0.064*	6
20 h	4.082 ± 0.133*	6
1 day	3.917 ± 0.096*	6
2 days	3.632 ± 0.106	5
3 days	3.416 ± 0.176	5
5 days	3.474 ± 0.068	5
7 days	3.249 ± 0.129	5

Table 3.1 Changes in paired testis weight of adult rats at different times after receiving a single subcutaneous injection of 100 IU hCG.

Data represent mean±SE.
*$P<0.05$, compared with saline-injected control using one-way ANOVA followed by Dunnett's test.

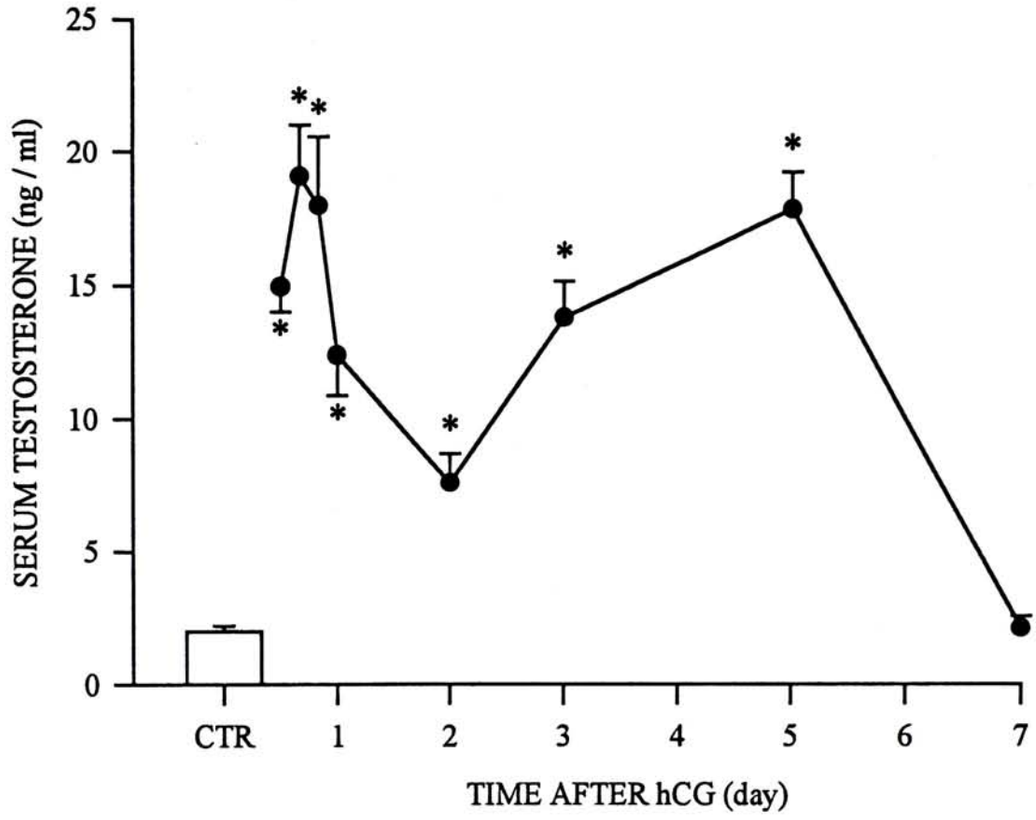

Figure 3.4 A biphasic change in serum testosterone concentration in adult rats after receiving a single subcutaneous injection of 100 IU hCG.

Data represent mean±SE of 5-6 animals per group.
*P<0.05, compared with saline-injected control (CTR) using one-way ANOVA followed by Dunnett's test.

value) of -0.132 was obtained. Subsequent statistical analysis indicated that there was no significant correlation (n=9, P>0.80) between these two parameters (Figure 3.5).

3.2 Effect of Leydig cell depletion by ethane dimethane sulphonate (EDS) on hCG-induced endothelial cell proliferation in adult rat testes

3.2.1 Testicular histology

In adult rats, injection of EDS resulted in a marked reduction in the number of testicular interstitial cells within 4 to 5 days after treatment (Figure 3.6). This was indicated by fewer numbers of cells and enlarged spaces in the testicular interstitium. Accompanying the loss of interstitial cells, the endothelium lining the testicular vasculature failed to respond to hCG stimulation with an increased number of cells expressing PCNA immunoreactivity (Figure 3.6).

3.2.2 Changes in the number of PCNA-positive endothelial cells

Under basal condition (without hCG stimulation), injection of EDS or the vehicle (i.e. DMSO) alone had no significant effect on the number of PCNA-positive endothelial cells in adult rat testes when compared with the control (Figure 3.7). However, prior EDS treatment completely abolished the hCG-induced increase in PCNA-positive endothelial cell number that was present in normal testes at 1 and 2 days post-hCG (Figure 3.7). The density of PCNA-positive endothelial cells in the testes of EDS-treated animals injected with hCG was not significantly different from that found under basal condition in normal, EDS-treated or DMSO-injected rats. In EDS-treated animals, the failure of PCNA-positive endothelial cell number to increase in response to hCG did not appear to be related to the vehicle injection since DMSO had no significant (P>0.05) effect on the basal or the hCG-induced increase in the

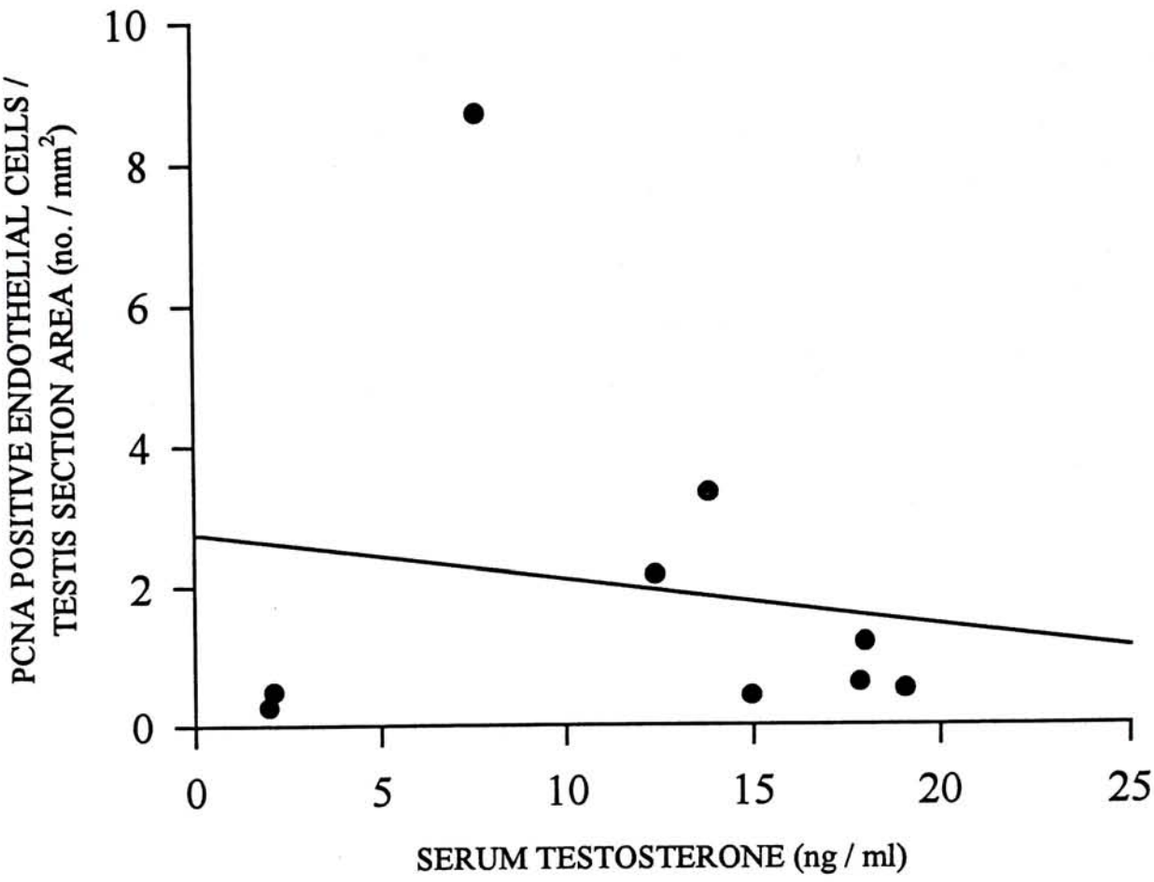

Figure 3.5 Correlation of the average PCNA-positive endothelial cell number/unit testis section area with the corresponding average serum testosterone levels in adult rats at different times after receiving a single subcutaneous injection of 100 IU hCG. (n=9, r = -0.132, P>0.80).

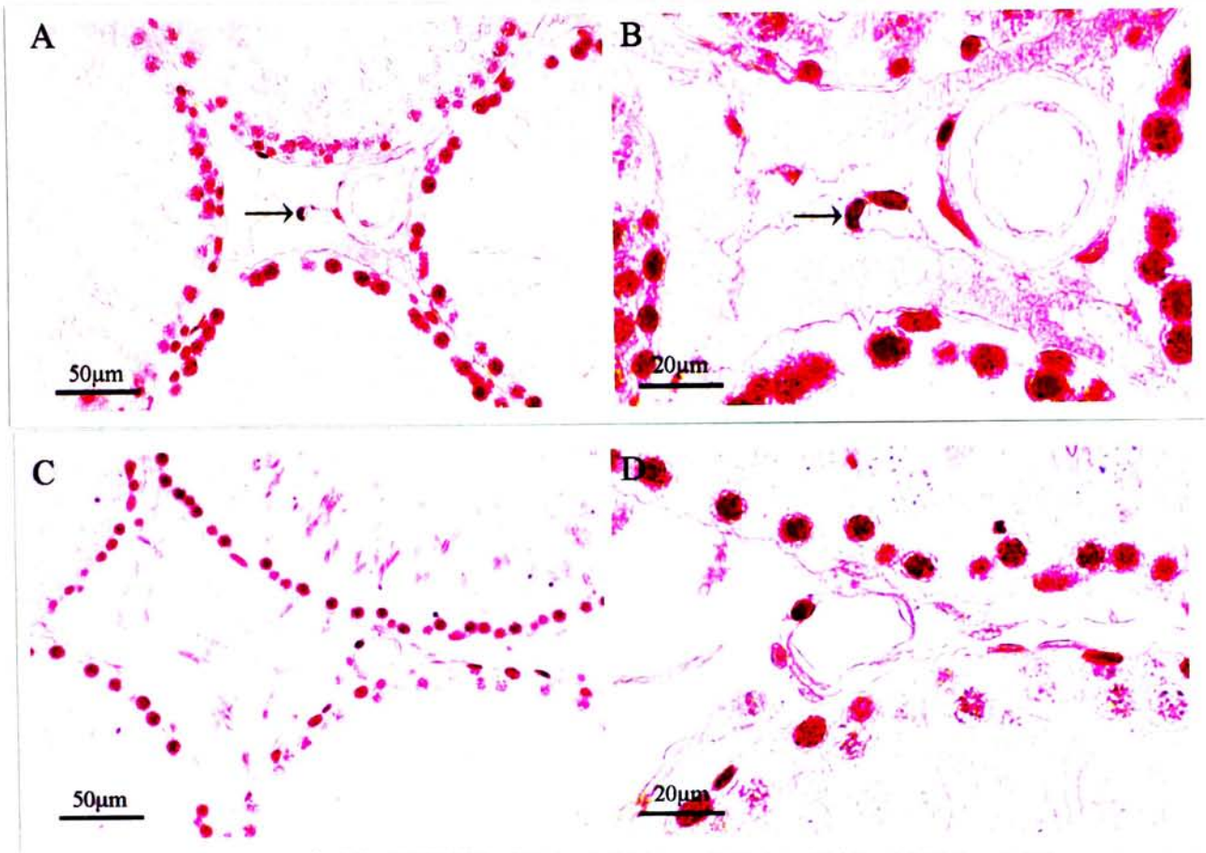

Figure 3.6 Representative PCNA immunostaining in testes of adult rats that received ethane dimethane sulphonate (EDS) 3 days before being injected with a single dose of saline (A & B) or 100 IU hCG (C & D) and studied 2 days post-hCG. Note that in both (A & B) and (C & D), there were very few interstitial cells after EDS treatment. The hCG-induced increase in the number of PCNA-positive endothelial cells as shown in Figure 3.1 could no longer be observed in the testes of EDS-treated rats (shown in C & D).

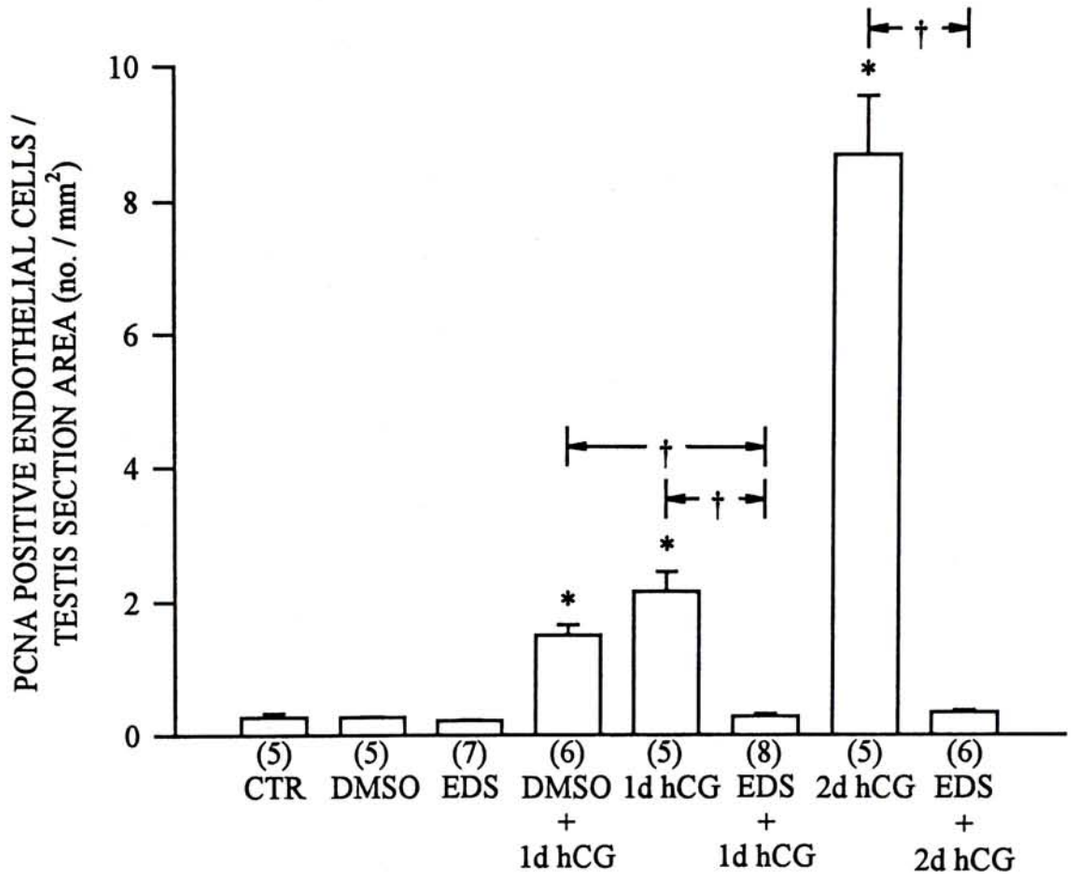

Figure 3.7 Effect of Leydig cell depletion on hCG-induced endothelial cell proliferation. Number of PCNA-positive endothelial cells (per unit section area) was determined in the testes of adult rats that received i.p. injection of ethane dimethane sulphonate (EDS) or the vehicle - dimethyl sulphoxide (DMSO) 3 days prior to being given a single subcutaneous injection of hCG and studied at 1 day and 2 days post-hCG.

Data represent mean±SE with the number of animals given in parentheses. *$P<0.05$, compared with saline-injected control (CTR) and
†$P<0.05$, compared with the corresponding "EDS-treated + hCG-injected" group, using one-way ANOVA followed by Student-Newman-Keuls test.

number of PCNA-positive endothelial cells when compared with the corresponding groups of normal rats injected with saline (control) or hCG (1 day hCG) alone.

3.2.3 Changes in serum testosterone concentration and testis weight

As expected, prior treatment of adult rats with EDS to destroy the Leydig cells resulted in a marked decrease in serum testosterone to very low levels that were approaching the detection limit of the radioimmunoassay (Figure 3.8). In these animals, their serum testosterone levels failed to show any significant changes even at 24 and 48 hours after receiving a subcutaneous injection of 100 IU hCG. When only the EDS-treated animals (with or without receiving hCG injection) were compared with either the saline-injected control or the vehicle (DMSO)-injected group (using one-way ANOVA followed by Student-Newman-Keuls test), their serum testosterone levels were shown to be significantly lower ($P<0.05$). However such statistical differences were loss when other treatment groups from the same experiment were included in the statistical analysis (Figure 3.8). In line with earlier findings, a significant increase in serum testosterone was observed at 1 and 2 days post-hCG in those animals with an intact population of Leydig cells (Figure 3.8). Prior injection of DMSO (i.p.) in these animals had no effect ($P>0.05$), if not a stimulatory one, on the serum testosterone response to hCG.

In EDS-treated rats, their testes weight did not differ significantly in comparison with the control when examined at 4 to 5 days after treatment. However unlike the group of normal or DMSO-injected animals, their testes weight failed to exhibit any significant changes or sign of an increase, 24 hours after receiving a subcutaneous injection of 100 IU hCG (Table 3.2).

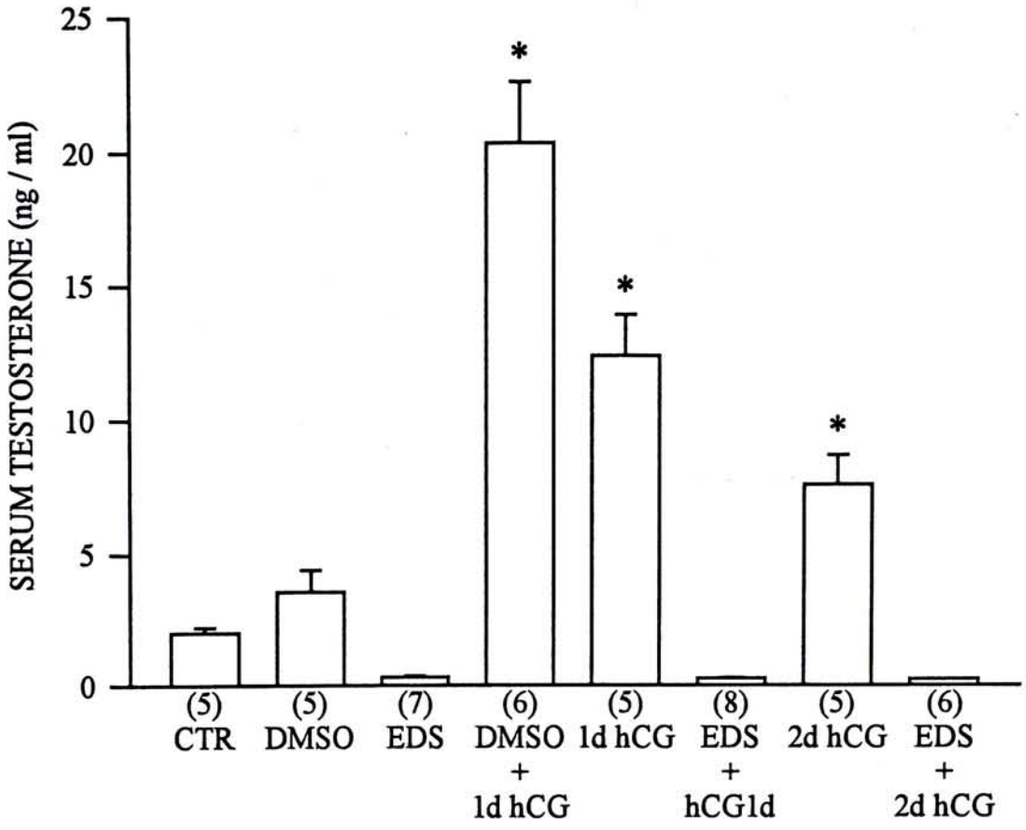

Figure 3.8 Effect of ethane dimethane sulphonate (EDS) or the vehicle (DMSO) treatment on the serum testosterone concentrations in adult rats that were subsequently given a single subcutaneous injection of 100 IU hCG (or saline) and studied at 1 and 2 days post-HCG.

Data represent mean±SE with the number of animals given in parentheses.
*P<0.05, compared with saline-injected control (CTR) using one-way ANOVA followed by Dunnett's test.

Treatment	Paired Testis Weight (gram)	No. of Animals
Control	3.416 ± 0.080	5
DMSO	3.525 ± 0.130	5
EDS	3.475 ± 0.100	7
DMSO + 1 day hCG	3.752 ± 0.066†	5
1 day hCG	3.917 ± 0.096*†	6
EDS + 1 day hCG	3.270 ± 0.102	7
2 day hCG	3.632 ± 0.106	5
EDS + 2 day hCG	3.473 ± 0.091	6

Table 3.2 Effect of pre-treating adult rats 3 days earlier with ethane dimethane sulphonate or the vehicle (DMSO) alone followed a single subcutaneously injection of 100 IU hCG (or saline) on the paired testis weight measured at 1 and 2 days post-hCG.

Data represent mean±SE.
*$P<0.05$, compared with saline-injected control using one-way ANOVA followed by Dunnett's test, and
†$P<0.05$, compared with "EDS + 1 day hCG" treatment group using one-way ANOVA followed by Student-Newman-Keuls test.

3.3 Effect of Leydig cell suppression by testosterone-filled subcutaneous silastic implants on hCG-induced endothelial cell proliferation in adult rat testes

3.3.1 Changes in serum testosterone concentration, testis weight, and testicular intertubular area

The two doses of testosterone implant were chosen such that the 3 cm implant would achieve normal physiological levels of serum testosterone but fail to provide adequate hormonal support for normal spermatogenesis, and the 25 cm implant would maintain near normal levels of spermatogenesis with a supraphysiological concentration of serum testosterone. These were well illustrated by the present results on the serum testosterone and the testis weight.

Serum testosterone levels in the 3 cm-implant group were almost identical to those of the age-matched control, and no statistical differences could be demonstrated between the two groups (Table 3.3). In animals bearing the 25 cm implant, their serum testosterone levels were significant higher ($P<0.05$) and gave a mean value of about 5-6 times that of the age-matched control or the 3 cm-implant group. Animals receiving exogenous testosterone implants (3 cm or 25 cm) showed a reduced serum testosterone response to hCG stimulation (Table 3.3). In the age-matched control not bearing any implants, serum testosterone was significantly elevated ($P<0.05$) above basal by about 3-fold at 48 hours after receiving a single s.c. injection of 100 IU hCG. In the 3 cm-implant group, hCG did not produce a significant rise in serum testosterone to levels above the basal. In the 25 cm-implant group, serum testosterone increased significantly ($P<0.05$) in response to hCG stimulation, but the increment was less than that observed in the age-matched control and represented only a 28% rise above the basal value found in the same treatment group (Table 3.3).

	Control		Treatment Groups			
Testosterone Implant	No Implant		3 cm		25 cm	
Subcutaneous Injection	saline	hCG	saline	hCG	saline	hCG
Paired Testis Weight (gram)	3.473 ± 0.092	3.632 ± 0.106	2.151 ± 0.146*	2.327 ± 0.089*	3.098 ± 0.119*	3.435 ± 0.081†
Percentage testis section area occupied by intertubular tissues (%)	14.04 ± 0.91	15.19 ± 0.55	16.80 ± 1.26	25.31 ± 2.28*,†	15.48 ± 1.16	17.40 ± 1.97
Serum Testosterone (ng/ml)	2.0 ± 0.2	7.6 ± 1.1†	2.1 ± 0.2	2.9 ± 0.4*	11.5 ± 0.6*	14.7 ± 1.1*,†
No. of Animals	5	5	6	6	6	6

Table 3.3 Effect of chronic Leydig cell suppression followed by acute hCG stimulation on the testis weight, percentage testis section area occupied by intertubular tissues, and serum testosterone concentration in adult rats. Chronic Leydig cell suppression was achieved by an 8-week treatment with either a low dose (3 cm) or a high dose (25 cm) of testosterone-filled subcutaneous implants. At the end of 8 weeks, the animals were given a single subcutaneous injection of hCG (100 IU) and studied 2 days post hCG.

Data represent mean±SE.
*$P<0.05$, compared with the corresponding saline- or hCG-injected group bearing no implants, and † $P<0.05$, compared with the corresponding saline-injected group receiving the same length of implant or no implant, using one-way ANOVA followed by Student-Newman-Keuls test.

In animals bearing the testosterone implants, the fall in testis weight mainly represented the loss of spermatogenic activity in the testes. In both 3 cm- and 25 cm-implant groups, the testis weight decreased significantly ($P<0.05$), and reached 61.9% and 89.2% of the value found in the age-matched control, respectively (Table 3.3). Following the injection of hCG, there was a tendency for the testis weight to increase in the normal as well as the testosterone-implanted groups. However, only those animals bearing the 25 cm implant responded with a significant increase ($P<0.05$) in testis weight above the basal found within the same treatment group or to a value which was insignificantly different from that in the age-matched control exposed to the same treatment (Table 3.3).

Since the testosterone implants affected the spermatogenic activity in adult rat testes, the size of the seminiferous tubules might decrease and so would be the area occupied by the tubular tissue. This would in turn affect the cross-sectional area of the testis upon which the quantification of PCNA-positive endothelial cell number was based. In an attempt to correct for this, the percentage of testis section area occupied by the intertubular tissue was determined in this experiment for the control and treatment groups. The intertubular area represents where the blood vessels are situated and it should be less affected by changes in the levels of spermatogenic activity within the seminiferous tubules. This might provide a better basis for expressing the surface density of PCNA-positive endothelial cells in testis sections.

Results indicated that under basal condition, there were no statistical differences among the age-matched control and the treatment groups in terms of the percentage testis section area occupied by the intertubular tissue (Table 3.3). Nevertheless the values from these three groups of animals could be ranked in the ascending order of control < 25 cm-implant group < 3 cm-implant group. This might

follow the expected trend of decreasing spermatogenic activity (or decreased seminiferous tubular size) in these animals. Following the exposure to hCG, the intertubular area showed a tendency to increase. In the 3 cm-implant group, it reached a significantly higher value when compared with the basal condition in the same treatment group or with the age-matched control exposed to the same dose of hCG (Table 3.3).

3.3.2 Changes in the number of PCNA-positive endothelial cells

Chronic Leydig cell suppression by exogenous testosterone released from subcutaneous silastic implants did not significantly affect the area density of PCNA-positive endothelial cells in the testes under basal condition (Figure 3.9). This holds true irrespectively of whether the cell density was expressed as per unit section area [Figure 3.9(A)] or as per unit intertubular area [Figure 3.9(B)]. Similarly, Leydig cell suppression did not appear to have any negative effect on the hCG-induced increase in the number of PCNA-positive endothelial cells in the testis. Although a low dose (3 cm) and a high dose (25 cm) of testosterone implants were used to achieve Leydig cell suppression, the results between the two treatment groups were not significantly different except when the number of PCNA-positive endothelial cells was expressed as per unit section area. In the 3 cm-implant group, this method of expression gave a significant higher value than the age-matched control and the 25 cm-implant group when compared after hCG treatment [Figure 3.9 (A)]. However such differences disappeared when the density of PCNA-positive cells was based on the area occupied by the intertubular tissue [Figure 3.9 (A)].

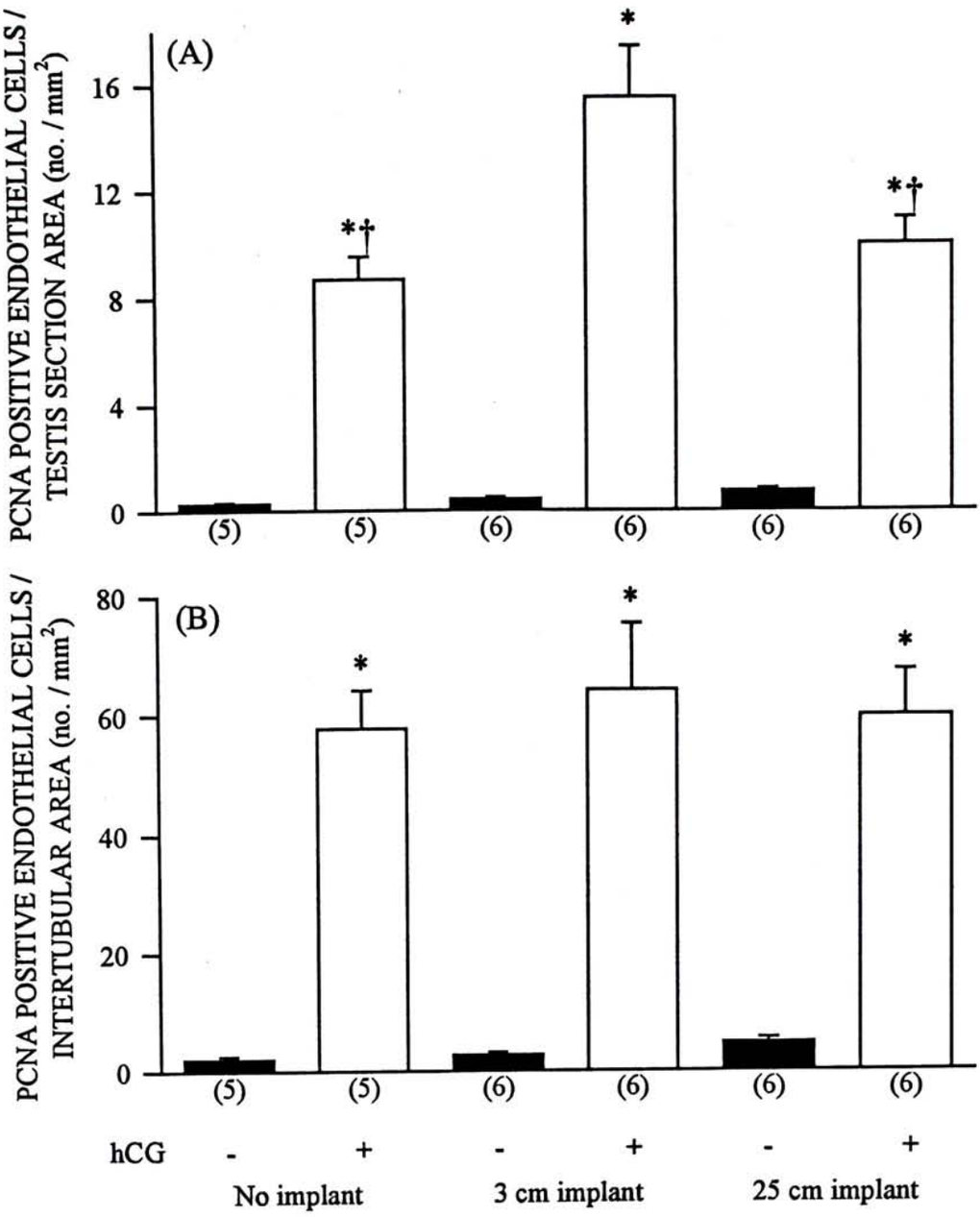

Figure 3.9 Effect of Leydig cell suppression on hCG-induced endothelial cell proliferation in adult rat testes. The number of PCNA-positive endothelial cells expressed either as per unit section area (A) or as per unit intertubular area (B), was determined in the testes of adult rats bearing 3 cm or 25 cm testosterone-filled implants for 8 weeks prior to being given a single subcutaneous injection of 100 IU hCG (or saline) and studied 2 days post-injection. Animals bearing no implants served as the control.

Data represent mean±SE with the number of animals indicated in parentheses. *$P<0.05$, compared with the corresponding saline-injected group (receiving no hCG), and †$P<0.05$, compared with the "hCG-injected 3 cm implant" group, using one-way ANOVA followed by Student-Newman-Keuls test.

3.3.3 Changes in the level of vascular endothelial growth factor (VEGF) immunoreactivity in the testis

In view of the earlier findings demonstrating a requirement for Leydig cells to mediate the hCG-induced increase in the number of PCNA-positive endothelial cells, the following study was carried out to determine how the suppression of Leydig cell function would affect the expression of a potent angiogenic factor - vascular endothelial growth factor, in the testis. In the control animals, VEGF immunostaining was localized mainly in the intertubular area within the cytoplasm of the interstitial cells [Figure 3.10 A & B], although Sertoli cells and vascular smooth muscle cells also contained some immunoreactivity. In animals bearing the testosterone implants, the interstitial cell population regressed considerably, and most of the VEGF immunoreactivity was lost from the intertubular area except the one present in vascular smooth muscle cells [Figure 3.10 C-F]. In comparison, the expression of VEGF in Sertoli cells had apparently increased in those animals bearing the testosterone implants.

3.4 Effect of testicular macrophage activation by polystyrene latex beads on hCG-induced endothelial cell proliferation in adult rat testes

3.4.1 Testicular histology

Intratesticular injection of polystyrene latex beads resulted in their accumulation in the cytoplasm of the testicular macrophages. As a result, the nuclei of these cells were pushed to one side. This phenomenon was recorded at 4-5 days after treatment [Figure 3.11(A)].

When the latex beads-induced testicular macrophage activation was combined with the stimulation by hCG, a marked increase in endothelial cell proliferation was

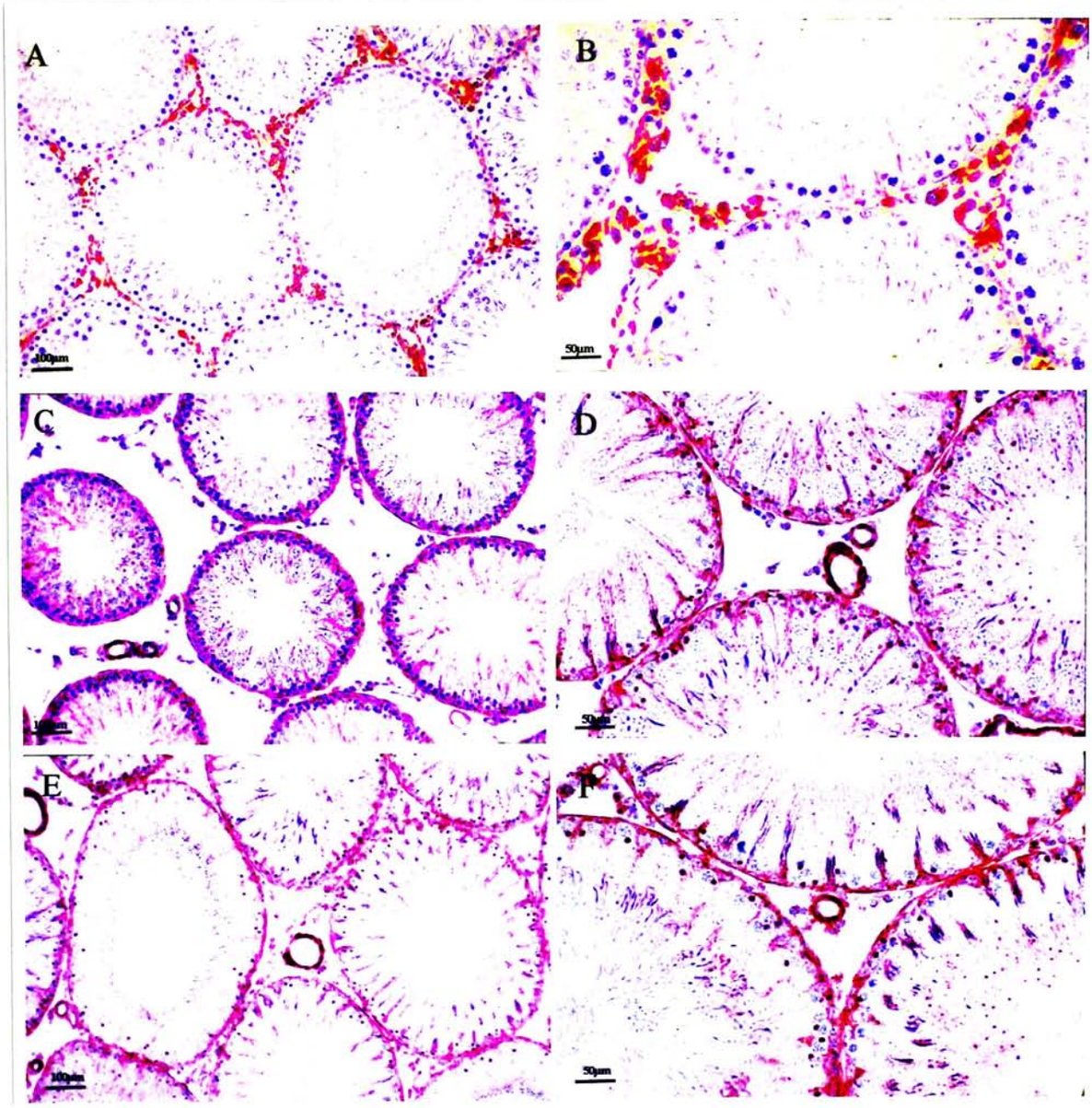

Figure 3.10 Immunohistochemical staining for vascular endothelial growth factor (VEGF) in the testes of normal adult rats (A & B) and adult rats that received subcutaneous testosterone-filled silastic implants of 3 cm (C & D) or 25 cm (E & F) in length for 8 weeks. In normal testes (A & B), most of the specific staining for VEGF was found within the cytoplasm of interstitial cells. In animals bearing testosterone implants of either 3 cm or 25 cm in length, many of the interstitial cells had regressed and the VEGF immunostaining was found mainly inside the Sertoli cells within the seminiferous tubules.

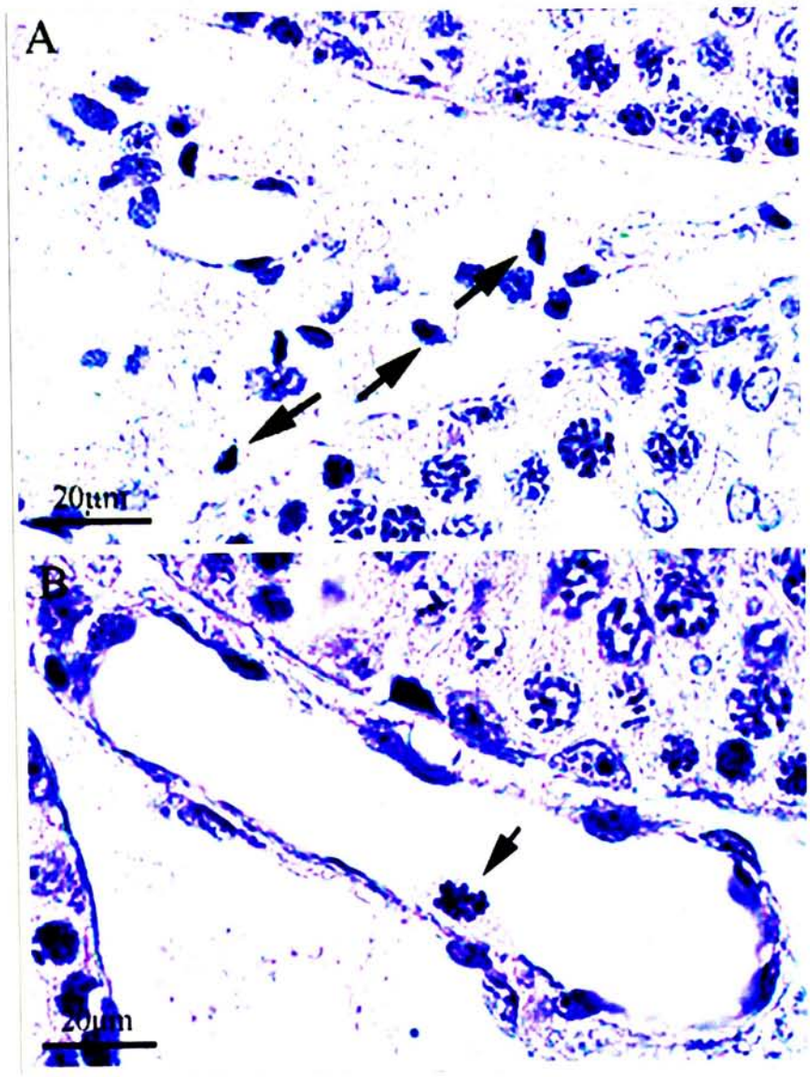

Figure 3.11 Testis sections (stained with haematoxylin) from adult rats that received intratesticular injection of polystyrene latex beads 3 days prior to being given a single subcutaneous injection of 100 IU hCG and studied 2 days post-hCG. (A) Under high magnification (1000x), testicular macrophages (indicated by arrows) were seen heavily laden with polystyrene latex beads and their nuclei were displaced to one side of the cells. (B) Macrophage activation combined with hCG stimulation resulted in a marked stimulation of endothelial cell proliferation. The arrow points to an endothelial cell undergoing mitotic division.

observed. In testis sections, the number of endothelial cells exhibiting the mitotic figure became a lot more abundant [Figure 3.11(B)], and this was also accompanied by a large increase in the number of PCNA-positive endothelial cells (data presented below).

3.4.2 Changes in the number of PCNA-positive endothelial cells

In the study of testicular macrophage activation, latex beads were injected unilaterally into the right testis while the contralateral testis was injected with sterile saline and used as the sham-operated control. Intratesticular injection of saline alone induced a significant increase ($P<0.05$) in the number of PCNA-positive endothelial cells, and the effect appeared to subside with the duration after treatment (Figure 3.12). Thus in the saline-injected testes not exposed to hCG stimulation, the density of PCNA positive endothelial cells increased above the basal value of 0.22 cell/mm^2 in normal control testes to 3.28 cells/mm^2 4 days after intratesticular injection, before it fell to 2.74 cells/mm^2 at 5 days post-injection (Figure 3.12). Such increases were similarly observed after the intratesticular injection of latex beads alone in the absence of hCG stimulation, and the effect also appeared to decrease with time after treatment with 5.54 cells/mm^2 and 2.41 cells/mm^2 at 4 and 5 days post-injection, respectively. Intratesticular injection of latex beads induced a significantly ($P<0.05$) larger increase in the density of PCNA-positive endothelial cells than saline injection when examined at 4 days after treatment [Figure 3.12(A)], but the difference disappeared on the 5th day [Figure 3.12(B)].

When hCG-induced endothelial cell proliferation in adult rat testes was examined after testicular macrophage activation by the injection of latex beads, a significant interaction between the two treatments was observed. At 2 days post-

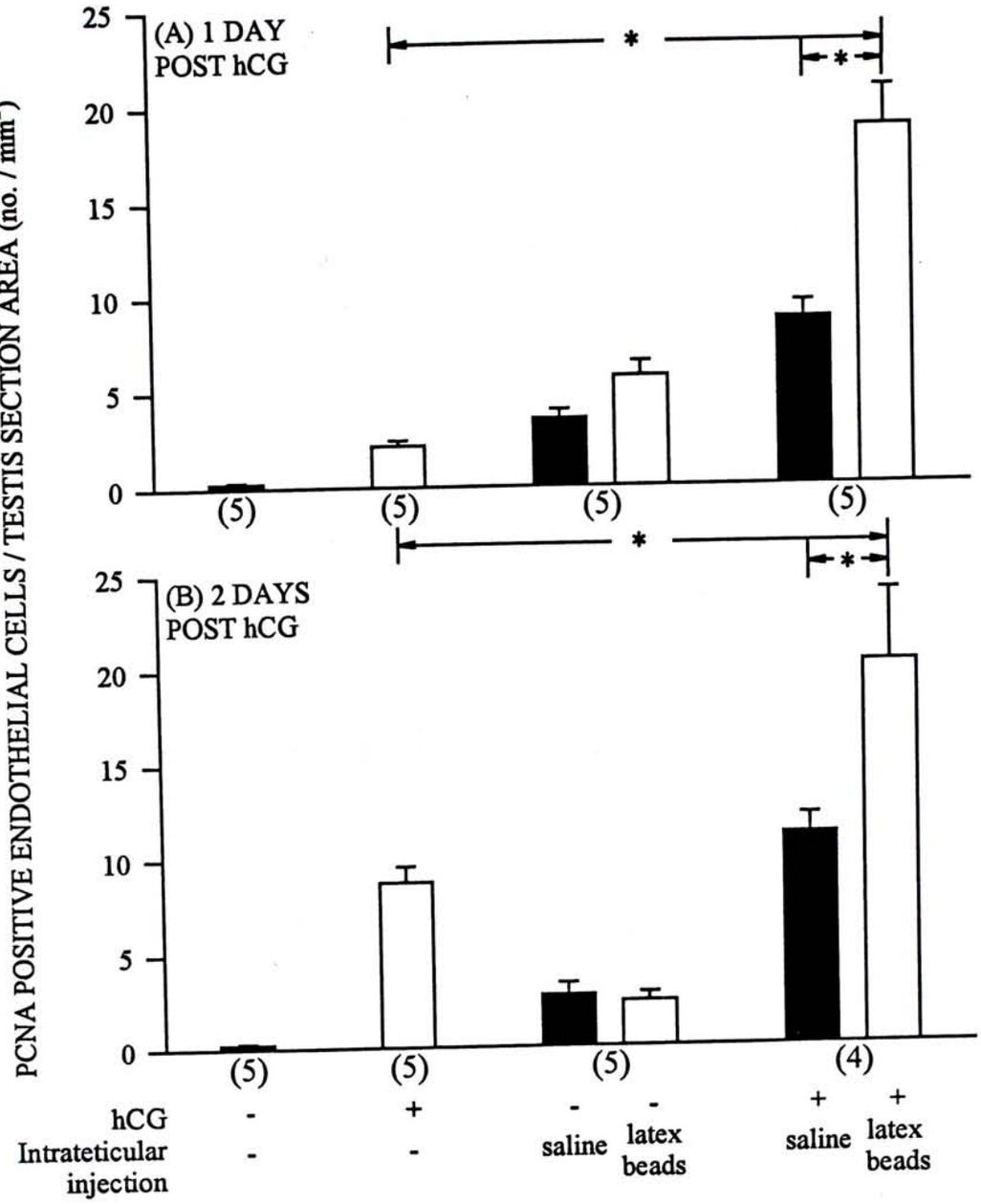

Figure 3.12 Effect of testicular macrophage activation by latex beads on hCG-induced endothelial cell proliferation in adult rat testes. Number of PCNA-positive endothelial cells (per unit section area) was determined in the testes of adult rats that received an intratesticular injection of polystyrene latex beads (2% in 150 µl sterile saline) into the right testes and sterile saline into the left testes (sham-operated control) 3 days prior to being given a single subcutaneous injection of 100 IU hCG and studied at 1 day (A) and 2 days (B) post-hCG.

Data represent mean±SE with the number of animals given in parentheses.
*$P<0.05$, using one-way ANOVA followed by Student-Newman-Keuls test.

hCG, the density of PCNA-positive endothelial cells increased from 8.69±0.87 cells/mm^2 (mean±SE) for hCG stimulation alone and 2.41±0.45 cells/mm^2 for latex beads-induced macrophage activation alone to a value of 20.3±3.75 cells/mm^2 for the combined treatment of the two [Figure 3.12(B)]. Similar interaction with an even greater increase above the individual effects was noted 1 day after the injection of hCG [Figure 3.12(A)]. Since the combined effect of the two treatments was approaching twice the sum of their individual effects, the data strongly suggested a synergistic interaction between hCG stimulation and macrophage activation in inducing endothelial cell proliferation in adult rat testes. Little or no synergism appeared to be present when hCG stimulation was combined with the sham-operation of injecting saline intratesticularly. At 2 days post-hCG, the density of PCNA-positive endothelial cells in saline-injected and hCG-stimulated testes was 11.17±1.03 cells/mm^2, which was roughly the sum of the individual effect due to hCG stimulation (8.69±0.87 cells/mm^2) and intratesticular saline injection (2.74±0.65 cells/mm^2) alone [Figure 3.12(B)].

3.4.3 Changes in testis weight and serum testosterone concentration

Intratesticular injection of latex beads or saline did not result in any significant changes in testis weights, although the values at day 4 post-injection (1.795±0.076 g for saline-injected and 1.975±0.170 g for latex bead-injected; mean±SE) were slightly higher ($P>0.05$) than those recorded at day 5 (1.796±0.042 g for saline-injected and 1.697±0.079 g for latex bead-injected) (Table 3.4). The intratesticular injection also appeared to produce larger variations in testis weight and thus mask the acute effect of hCG in producing a significant increase in testis weight at 1 day post-hCG. In animals not receiving any intratesticular injection, a significant increase in testis

Subcutaneous Injection	Intratesticular Injection	Unpaired Testis Weight (gram)	Serum Testosterone (ng/ml)
saline (control)	-	1.708 ± 0.040 (n=5)	5.0 ± 1.1 (n=5)
1 day hCG	-	1.958 ± 0.048* (n=6)	10.0 ± 1.8* (n=5)
1 day saline	saline	1.795 ± 0.076 (n=5)	8.3 ± 1.9 (n=5)
	latex beads	1.975 ± 0.170 (n=5)	
1 day hCG	saline	1.855 ± 0.093 (n=5)	14.1 ± 2.2* (n=5)
	latex beads	2.015 ± 0.232 (n=5)	
2 days hCG	-	1.816 ± 0.053 (n=5)	5.8 ± 0.8 (n=5)
2 days saline	saline	1.796 ± 0.042 (n=5)	4.6 ± 0.7 (n=5)
	latex beads	1.697 ± 0.079 (n=5)	
2 days hCG	saline	1.652 ± 0.057 (n=5)	5.2 ± 0.6 (n=5)
	latex beads	1.561 ± 0.078 (n=5)	

Table 3.4 Effect of testicular macrophage activation followed by acute hCG stimulation on the testis weight and serum testosterone concentration in adult rats. Testicular macrophage activation was induced unilaterally by injecting polystyrene latex beads into the right testis. Sterile saline was injected into the left testis which served as the sham-operated control. Three days later, 100 IU hCG was injected subcutaneously and the animals were studied at 1 day and 2 days post-hCG.

Data represent mean±SE. No statistical difference in the testis weight was observed. *$P<0.05$, compared with the control using one-way ANOVA followed by Dunnett's test.

weight was recorded at 1 day post-hCG (1.958±0.048 g for hCG-injected versus 1.708±0.040 g for the control), and in animals with saline- (1.855±0.093 g) and latex beads-injected (2.015±0.232 g) testes, the increases in weight were not statistically significant (Table 3.4). It was also noted that at 2 days post-hCG, those animals that had previously been given intratesticular injections of saline and latex beads appeared to have a lower testis weight when compared with the control, although their differences were not statistically significant.

In the treatment groups, intratesticular injection of latex beads and saline (into the right and left testis respectively) did not produce any significant systemic endocrine changes in terms of serum testosterone levels when examined at 4-5 days after treatment. Their serum testosterone levels (8.3±1.9 ng/ml and 4.6±0.7 ng/ml, respectively) did not differ significantly from those present in the normal control (5.0±1.1 ng/ml) (Table 3.4). Subcutaneous injection of 100 IU hCG in these animals produced a significant ($P<0.05$) rise in serum testosterone when examined at 24 hours (14.1±2.2 ng/ml) but not 48 hours (5.2±0.6 ng/ml) post-hCG. Their serum testosterone responses did not differ significantly from those determined at the corresponding times of 24 (10.0±1.8 ng/ml) and 48 hours (5.8±0.8 ng/ml) post-hCG in normal rats (without receiving any intratesticular injection).

3.5 Effect of testicular macrophage depletion by liposome-entrapped Cl_2MDP treatment on hCG-induced endothelial cell proliferation in adult rat testes

3.5.1 Testicular histology

Intratesticular injection of Cl_2MDP-lp resulted in the depletion of testicular macrophages which progressed from the centre to the surface of the testis (Figure 3.13). In the present study, these animals were used 14 days post-Cl_2MDP-lp

injection. This duration of treatment had previously been shown to produce the maximum decrease in the number of testicular macrophages to reach about 14% of the normal control values (Bergh, Damber & van Rooijen, 1993).

In normal adult rats, subcutaneous injection of 100 IU hCG resulted in a marked infiltration of polymorphonuclear leucocytes (PMN) into the testes, however the changes would have completely subsided by 32 hours post-hCG. In animals that received intratesticular injection of Cl_2MDP-lp to deplete the testicular macrophages, the infiltration of PMN could still be observed at 48 hours post-hCG when many PMN were seen adhering to the wall of blood vessels and lying within the testicular interstitium (Figure 3.14).

3.5.2 Changes in the number of PCNA-positive endothelial cells

Bilateral intratesticular injection of Cl_2MDP-lp to deplete the testicular macrophages did not result in any significant changes in the density of PCNA-positive endothelial cells in adult rat testes when examined under the basal state (in the absence of hCG stimulation). The comparisons were made 16 days after the injection and they showed no significant differences among the Cl_2MDP-lp-injected animals, the sham-operated (receiving intratesticular injection of PBS-lp) and the normal (receiving no injection) control groups (Figure 3.15). However, Cl_2MDP-lp treatment (given 14 days earlier) significantly potentiated the effect of hCG in inducing an increase in the number of PCNA-positive endothelial cells in adult rat testes when examined at 2 days post-hCG. The density of PCNA positive endothelial cells in the Cl_2MDP-lp-injected testes exposed to hCG (13.16±2.25 cells/mm^2; mean±SE) was significantly higher than that present in the PBS-lp-injected (7.10±0.82 cells/mm^2) and

Figure 3.13 Intratesticular injection of Cl_2MDP-lp caused the depletion of macrophages to progress from the centre to the surface of the testis. In (A) (without counterstaining), it shows the junction between two regions of the testis with and without trypan blue-labelled testicular macrophages. In (B), it shows a testis two weeks after Cl_2MDP-lp treatment when the testicular macrophages (indicated by arrows) were mostly confined to the region immediately beneath the testicular capsule.

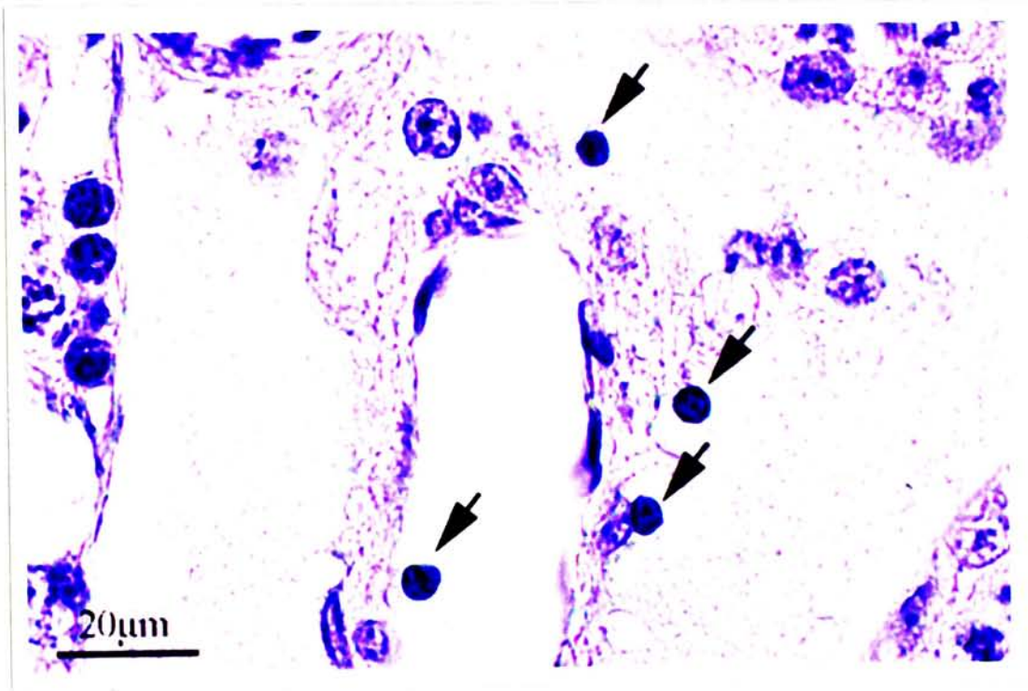

Figure 3.14 Testis sections (stained with haematoxylin) from adult rats that received intratesticular injection of Cl_2MDP-lp 2 weeks prior to being given a single subcutaneous injection of 100 IU hCG and studied 2 days post hCG. An inflammation-like reaction of polymorphonuclear (PMN) leukocyte (indicated by arrows) infiltration was observed similar to the early response of normal testes to the same dose of hCG within 4-16 hours post-hCG. Some PMN leukocytes were found adhering to the endothelial lining of blood vessels while others had infiltrated into the testicular interstitium.

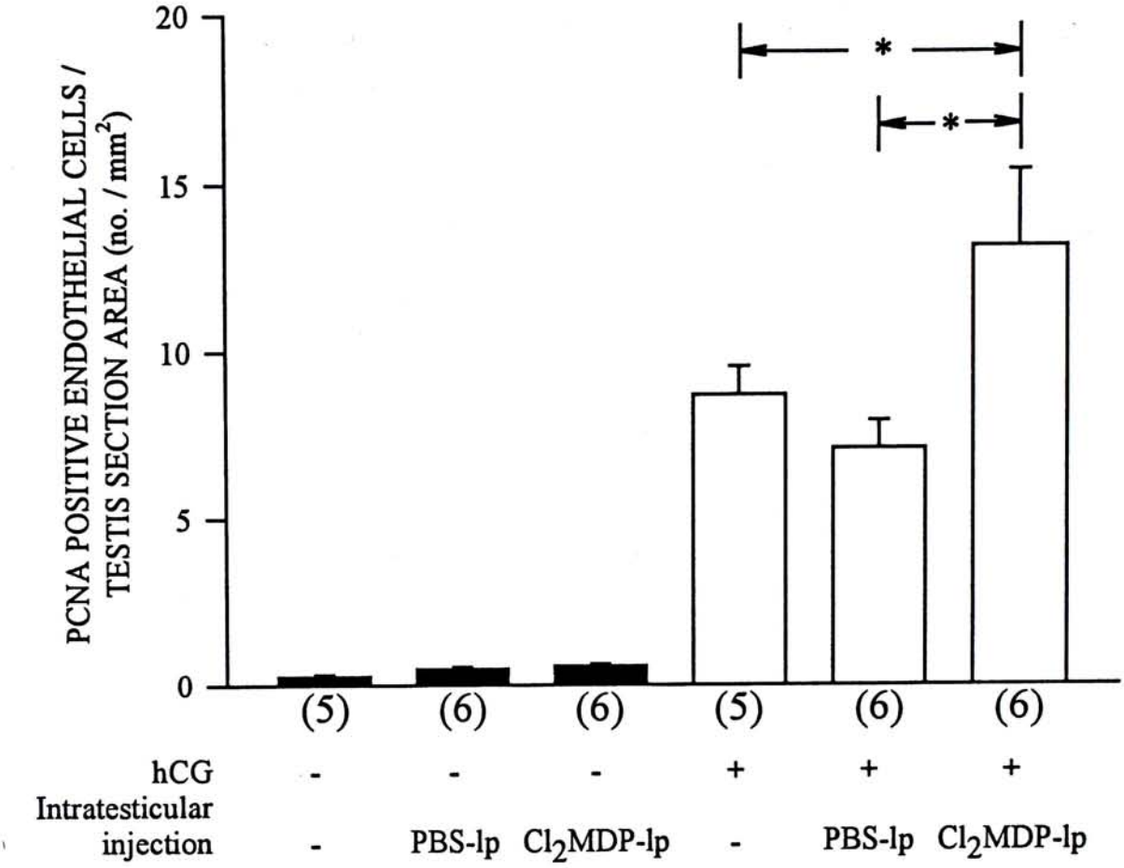

Figure 3.15 Effect of testicular macrophage depletion by Cl_2MDP-lp treatment on hCG-induced endothelial cell proliferation in adult rat testes. Number of PCNA-positive endothelial cells (per unit section area) was determined in the testes of adult rats that received intratesticular injection of Cl_2MDP-lp (~6 mg in 150 µl PBS) or PBS-lp (in the sham-operated control) 14 days prior to being given a single subcutaneous injection of 100 IU hCG (or saline) and studied 2 days post-hCG.

Data represent mean±SE with the number of animals given in parentheses.
*$P<0.05$, using one-way ANOVA followed by Student-Newman-Keuls test.

normal (8.69±0.87 cells/mm^2) testes stimulated by the same dose of hCG (Figure 3.15).

3.5.3 Changes in testis weight and serum testosterone

Intratesticular injection of Cl_2MDP-lp or PBS-lp did not produce any significant alteration in testis weight when compared with the age-matched control at 16 days after the treatment (Table 3.5). In these animals, their testis weight remained insignificantly affected even after they had received a subcutaneous injection of 100 IU hCG 2 days earlier, and compared against the corresponding treatment groups not exposed to hCG (Table 3.5). Similar findings were recorded when comparing the testis weight of normal rats (not receiving any intratesticular injection) with and without receiving the same dose of hCG.

Intratesticular injection of Cl_2MDP-lp or PBS-lp resulted in a fall in serum testosterone levels when comparisons were made against the normal control matched for age (Table 3.5). Between the Cl_2MDP-lp-injected group (1.0±0.3 ng/ml; mean±SE) and the control (5.0±1.1 ng/ml), the difference was statistical significant ($P<0.05$). When the Cl_2MDP-lp and PBS-lp injected groups were given a subcutaneous injection of 100 IU hCG, their serum testosterone levels (7.7±0.7 ng/ml and 7.8±1.4 ng/ml, respectively) became significantly elevated above the basal after 2 days and did not differ significantly from the values determined in the group of normal rats (5.8±0.8 ng/ml) similarly treated with the same dose of hCG.

3.6 Endothelial cell proliferation in rat testes during postnatal development

3.6.1 Changes in the number of PCNA-positive endothelial cells

	Control		Treatment Groups			
Intratesticular Injection	-		PBS-lp		Cl_2MDP-lp	
Subcutaneous Injection	saline	hCG	saline	hCG	saline	hCG
Paired Testis Weight (gram)	3.416 ± 0.080 (n=5)	3.632 ± 0.106 (n=6)	3.653 ± 0.104 (n=5)	3.801 ± 0.078 (n=5)	3.806 ± 0.050 (n=5)	3.646 ± 0.114 (n=5)
Serum Testosterone (ng/ml)	5.0 ± 1.1 (n=5)	5.8 ± 0.8 (n=5)	2.4 ± 0.7 (n=5)	7.8 ± 1.4* (n=5)	1.0 ± 0.3 (n=5)	7.7 ± 0.9* (n=5)

Table 3.5 Effect of testicular macrophage depletion followed by acute hCG stimulation on the testis weight and serum testosterone concentration in adult rats. Testicular macrophage was depleted by Cl_2MDP-lp treatment given 14 days before 100 IU hCG was injected subcutaneously, and the animals were studied at 2 days post-hCG.

Data represent mean±SE.
*$P<0.05$, compared with the corresponding saline-injected group using one-way ANOVA followed by Student-Newman-Keuls test.

When the number of PCNA-positive endothelial cells was expressed over per unit section area, the maximum value (6.4±1.6/mm^2; mean±SE) was found in 10-day old rats [Figure 3.16(A)]. Between 10 and 20-day old rats, this value dropped by about half (to 3.3±0.08/mm^2) before it continued to decline gradually to the lowest value found in 90-day old rats (0.27±0.06/mm^2). In view of the fact that the above expression could be influenced by the initiation of spermatogenesis in the seminiferous tubules leading to an enlargement of the tubular compartment and hence its contribution towards the total testis section area, comparisons were also made based upon the number of PCNA-positive endothelial cells per unit intertubular area. With this method of expression, maximum values were found in 10- to 30-day old rats before it gradually declined with age to reach the minimum value again in 90-day old rats [Figure 3.16(B)]. When statistical comparisons were made against the 90-day rats which was arbitrarily taken as the reference for adult animals, no significant differences in the number of PCNA-positive endothelial cells/unit intertubular area could be identified once the animals reached the age of 60-day old or above.

3.6.2 Changes in blood vessel density

The changes in testicular blood vessel density were examined among the different age groups and again there were differences in the pattern depending on whether they were expressed as number per unit testis section area or number per unit intertubular area (Figure 3.17). With the former method of expression, the blood vessel density was the highest in 10-day old rats (179.5±6.4 /mm^2; mean±SE) [Figure 3.17(A)]. Between 10- and 20-day old rats, the blood vessel density dropped by about half (92.5±6.6 /mm^2) before it stabilized at around 65-95/mm^2 in 30- to 90-day old rats. This pattern resembled that of the density of PCNA-positive endothelial cells

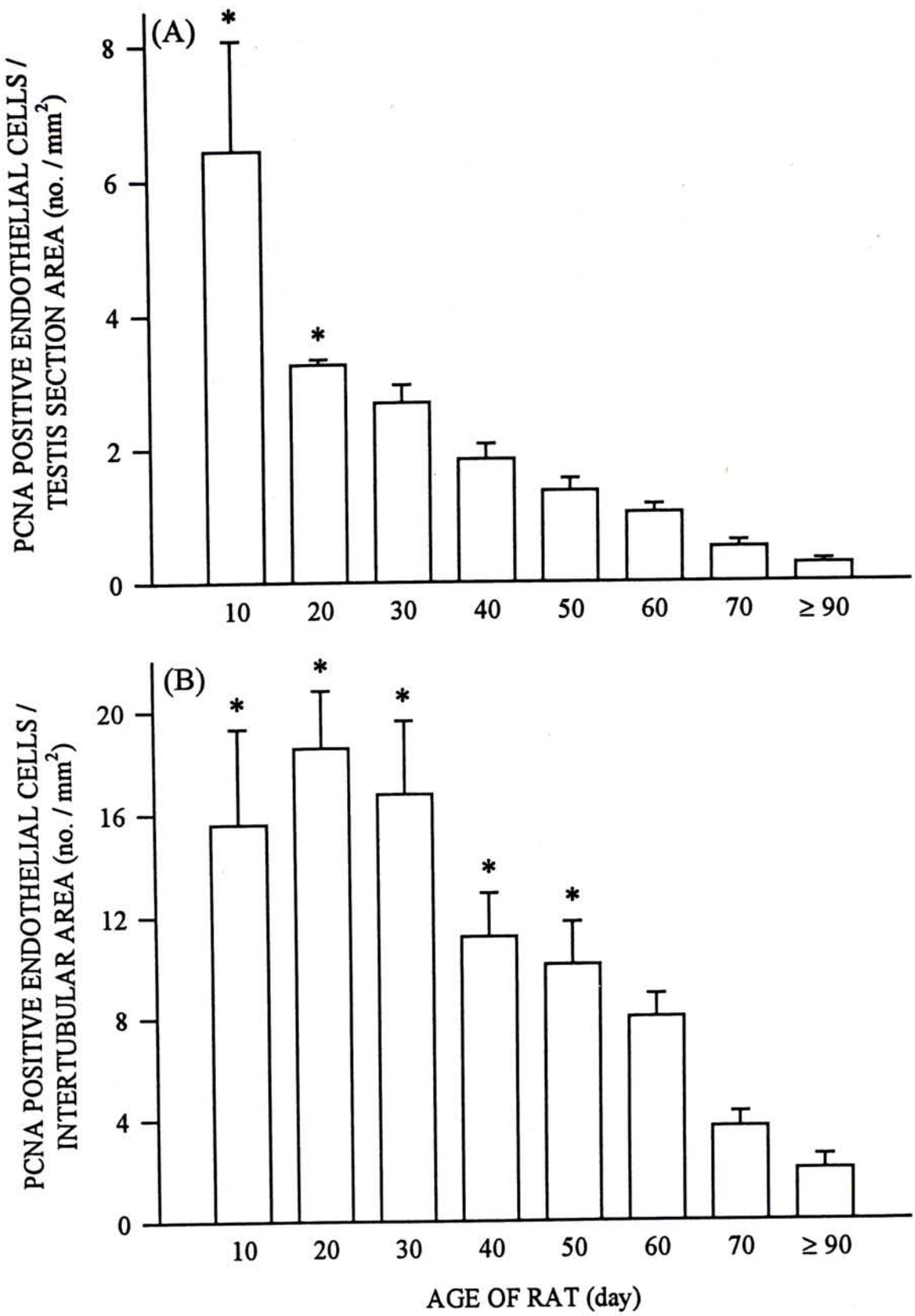

Figure 3.16 Changes in the number of PCNA-positive endothelial cells in the testes of rats during postnatal development from the age of 10 to 90 days old. The endothelial cell numbers were expressed either as per unit section area (A) or as per unit intertubular area (B).

Data represent mean±SE of 5-6 animals per group.
*$P<0.05$, compared with 90-day old rats using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test.

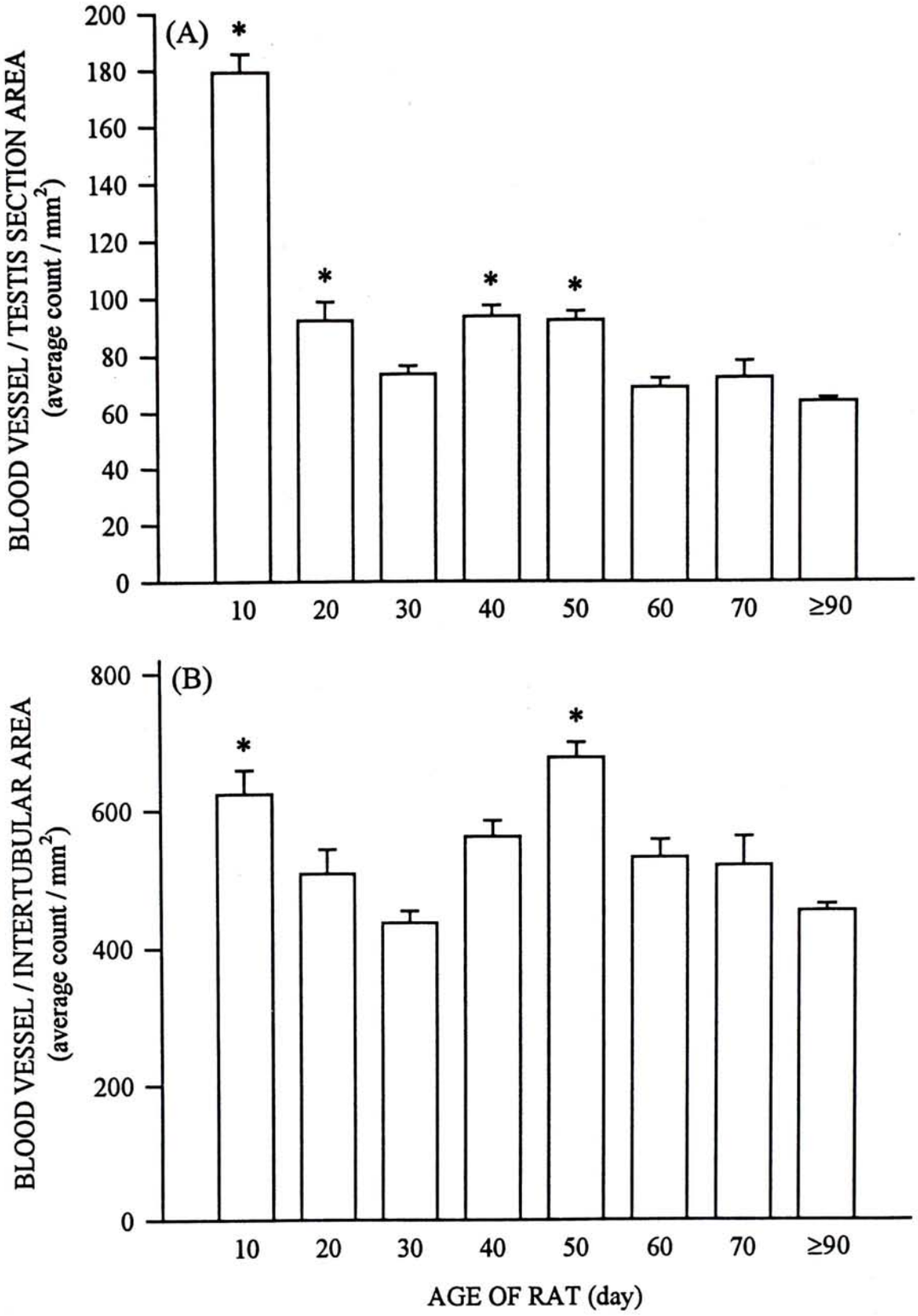

Figure 3.17 Changes in the testicular blood vessel density during postnatal development of the rats from the age of 10 to 90 days old. The blood vessel density was expressed either as per unit section area (A) or as per unit intertubular area (B).

Data represent mean±SE of 5-6 animals per group.
*$P<0.05$, compared with 90-day old rats using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test.

expressed on the same basis of per unit section area [Figure 3.16(A)]. On the other hand when the blood vessel density was expressed as number per unit intertubular area, there was a biphasic change with the first peak in 10-day old rats ($624\pm22/mm^2$) followed by a second peak in 50-day old rats ($678\pm22/mm^2$). Statistical analyses indicated that only the values at these two ages were significantly ($P<0.05$) higher than that in 90-day old rats.

3.6.3 Changes in serum testosterone concentration, testis weight and testicular intertubular area

Due to the difficulty in collecting enough sera for radioimmunoassay, serum testosterone concentration was not determined in 10-day old rats. Results indicated that there were no significant differences in serum testosterone concentration in 20- to ≥90-day old rats. The testis weights of younger animals (10- to 30-day old rats) were missing since their testes were fixed by whole body perfusion and fresh weights could not be measured. When comparing among the other age groups, the testis weight increased with the age of the animals until between 60- and ≥90-days old, it stabilized at the values found in adult animals (Table 3.6). Statistical tests indicated that there were no significant differences in the testis weight among the 60-, 70- and ≥90-day old rats. As for the percentage of testis section area occupied by intertubular tissue, the maximum value was found in 10-day old rats before it fell gradually to significantly ($P<0.05$) lower values found in 50- to ≥90-day old rats.

	10d	20d	30d	40d	50d	60d	70d	≥ 90d
Paired Testis Weight (gram)	-	-	-	1.699 ± 0.006 * (n=5)	2.665 ± 0.086 * (n=5)	3.628 ± 0.117 (n=5)	3.736 ± 0.114 (n=5)	3.416 ± 0.080 (n=5)
Percentage testis section area occupied by intertubular tissues (%)	28.74 ± 1.33* (n=6)	18.19 ± 1.39* (n=5)	16.82 ± 1.29 (n=5)	16.68 ± 0.59 (n=6)	13.63 ± 0.38 (n=6)	12.92 ± 0.84 (n=6)	13.88 ± 0.66 (n=6)	14.04 ± 0.91 (n=5)
Serum Testosterone (ng/ml)	-	3.3 ± 0.8 (n=5)	2.0 ± 0.3 (n=5)	1.8 ± 0.1 (n=5)	2.4 ± 0.3 (n=5)	2.0 ± 0.3 (n=5)	2.7 ± 0.3 (n=5)	3.2 ± 0.5 (n=5)

Table 3.6 Changes in paired testis weight, serum testosterone and percentage testis section area occupied by intertubular tissues in rats during postnatal development from the age of 10 to ≥90 days old.

Data is indicated as mean±SE.
*$P<0.05$, compared with ≥90 days old rats using one-way ANOVA followed by Student-Newman-Keuls test.

4. Discussion

In the present study, testicular angiogenesis was being investigated in response to a single injection of hCG and during postnatal development in the rat testes. The immunostaining for PCNA-labelled proliferating endothelial cells and blood vessel density were used as an indices of angiogenesis. Changes in blood vessel density were quantified using an image analysis software and related to the number of PCNA-positive endothelial cells per unit area.

4.1 hCG-induced endothelial cell proliferation and changes in blood vessel density

Using immunohistochemical staining for PCNA as a marker of proliferating cells, hCG was shown to induce a significant increase in the number of proliferating endothelial cells in adult rat testes between 24 and 72 hours after treatment (Au et al., 1996). Although PCNA is not exclusively a S-phase marker, a recent study using bromodeoxyuridine (BrdU) to label testicular endothelial cells in the S-phase of the cell cycle also reported a significant stimulation of endothelial cell proliferation by 50 IU hCG 24 hours after a single injection (Collin & Bergh, 1996). A detail time course study performed here revealed that following a single injection of hCG, the number of proliferating endothelial cells increased and then returned back to basal levels over a period of within 7 days, and the maximum increase was found 48 hours after treatment.

Whether the increased number of PCNA-positive endothelial cell per unit testis section area truly reflects an elevated level of testicular angiogenesis has been addressed in the present study by quantifying the blood vessel density in the testes of these animals. From the results, it can be seen that the peak of endothelial cell proliferation is followed 24 hours later by an increase in blood vessel density to reach

a maximum. The temporal relationship between the two changes suggests that the hCG-induced increase in the number of proliferating endothelial cells is followed later by the laying down of new blood vessels, thus finally cumulating in the appearance of more blood vessels per unit area within the testis. In addition, most of the PCNA-positive endothelial cells identified in the present study were found lining the venules and capillaries, and these are the sites where the sprouting of new blood vessels regularly occurs.

It should be noted that a single injection of hCG did not produce a sustained increase in the testicular blood vessel density as it reached its maximum after 3 days and then returned back to normal levels 7 days after treatment. This would imply that the formation of new blood vessels was accompanied later on by the dissolution of some of the new blood vessels. The loss of these vessels may involve endothelial cell apoptosis which is occasionally seen in normal testes and has been considered as one of the two major forces (the other being cell proliferation) responsible for the continuous remodelling of the testicular microvasculature (Collin & Bergh, 1996). Alternatively, as what has been found during the lutolysis of the corpus luteum, the newly formed blood vessels may regress by gradual foreshortening, rounding and finally detachment of endothelial cells without undergoing apoptosis (Augustin et al., 1995).

In the past, many studies have reported the use of the proliferation index (ratio of PCNA-positive endothelial cell nuclei/total number of endothelial cell nuclei counted × 100, i.e. percentage of positive cells) as a marker of angiogenesis (Goodger & Rogers 1993; Heron & Rakusan 1995; Kawanami, et al., 1995; Burke, Lehmann-Bruinsma & Powell 1995; Heron, et al., 1997). However, others have relied on the use of PCNA-positive endothelial cell number as a marker of vascular proliferation

(Soini, et al., 1996; Nanji, et al., 1994; Nanji & Tahan, 1996; Yamamoto, et al., 1997). In the present study, since the testis size was not very much affected by hCG stimulation as indicated by the relatively small changes in testis weight, it was therefore possible to assume that the total number of endothelial cells per unit section area remained relatively constant. Thus monitoring the changes in the number of PCNA positive endothelial cells per unit section area would yield almost identical information as using the proliferation index (as defined above).

The vascular effects of hCG on the testis has been well-documented (reviewed in the "Introduction"). In adult rats, the testes respond to a single injection of hCG with a decrease followed by an increase in blood flow, and an inflammation-like reaction of polymorphonuclear leukocytes infiltration and vascular permeability increase (Sharpe, 1979; Setchell & Sharpe, 1981; Sharpe & Cooper, 1983; Widmark, Damber & Bergh, 1986; see review by Bergh, Damber & Widmark, 1988). The last effect results in the accumulation of interstitial fluid which could explain why in the present study, the testis weight of these animals was significantly increased at 16-24 hours post-hCG. All the vascular changes induced by hCG do not occur immediately. The initial decrease in testicular blood flow and the start of the PMN infiltration begin 4-6 hours after hCG injection (Bergh, Damber & Widmark, 1988). Increases in testicular blood flow and vascular permeability are reported to occur 16-20 hours post-hCG (Setchell & Sharpe, 1981). These contrast the serum testosterone response which shows a marked increase as early as 2 hours post-injection (Hodgson & de Kretser, 1982). In the present study, significant increases in the area density of PCNA-positive endothelial cells and blood vessel density were observed at 1 day and 3 days post-hCG, respectively. These findings would suggest that the hCG-induced vascular changes do not result from a direct action of this hormone on the testicular

vasculature. In fact, no LH/hCG receptors have been found in testicular blood vessels or on the surface of vascular endothelial cells. Thus the vascular effects of hCG are likely to have been produced indirectly through the release of other mediators from within the testis, and time is needed for the generation of these mediators to cause the observed changes.

The present findings confirm that a single injection of 100 IU hCG induces a typical biphasic serum testosterone response, which is in line with a few earlier studies (Hodgson & de Kretser 1982; Hodgson & de Kretser 1984). However, correlation analysis indicated that there was no significant temporal relationship between the changes in serum testosterone levels and the density of PCNA-positive endothelial cells in testis section. The maximum increase in endothelial cell proliferation was observed 2 days after hCG stimulation when the serum testosterone was at its nadir between the two peaks. Thus the hCG-induced endothelial cell proliferation in the testis does not appear to have resulted from the increase in testosterone production by the Leydig cells.

4.2 Role of Leydig cells in hCG-induced endothelial cell proliferation in adult rat testis

The present study demonstrates that destroying the Leydig cells by EDS treatment completely abolishes the increase in the number of PCNA-positive endothelial cells induced by hCG. The fact that vehicle-DMSO treatment did not significantly affect the endothelial cell response to hCG would indicate that the effect due to EDS is specific. Furthermore, since EDS (or DMSO) has no effect on the number of PCNA-positive endothelial cells under basal state (without hCG stimulation), these cell proliferation may represent normal turnover of this cell type

within the testicular vasculature, and they are not influenced by hormonal changes such as low intratesticular and circulating levels of testosterone induced by EDS treatment.

The above findings are in line with the fact that Leydig cells represent the only cell type within the testis that express LH/hCG receptor. Also the Leydig cell is a source of several angiogenic factors including VEGF and bFGF (see 'Introduction'). In a study that examined the effect of testicular grafts in rabbits, it was found that only those testicular tissues containing interstitial cells would induce an elaborate growth of blood vessels around the grafts (Williams, 1949). More recently, the above study was repeated by transplanting interstitial cells or seminiferous tubule segments under the kidney capsule and observed the development of a prominent vascular network around the interstitial cell grafts but not the tubule grafts (Collin & Bergh, 1996). The authors of the latest study proposed that VEGF secreted by the Leydig cells together with other inflammation mediators are most likely involved in producing the hCG-induced endothelial cell proliferation in the testis. This would agree with the possible role of VEGF in mediating hormonally-regulated angiogenesis in other endocrine glands such as the ovary and the thyroid (reviewed in the 'Introduction').

Despite the evidence in support of the participation of Leydig cells in producing the hCG-induced endothelial cell proliferation, the role of VEGF remains unclear. In EDS-treated testes, although VEGF immunoreactivity is lost from the testicular interstitium, the ones present in Sertoli cells and vascular smooth muscle cells remain (Au *et al.*, 1997). Nevertheless, hCG-induced endothelial cell proliferation was completely abolished in EDS-treated testes. Thus it is unlikely that VEGF from other cellular sources within the testis (e.g. Sertoli cells, vascular smooth muscle cells) would be involved or could compensate for the deficiency resulted from

the removal of Leydig cells. Furthermore, in adult rats that received chronic treatment with the testosterone implants, the levels of hCG-induced endothelial cell proliferation in the testes remain indistinguishable from those present in normal animals despite a very low level of VEGF immunoreactivity present in these highly regressed Leydig cells. It can be argued that the loss of VEGF immunoreactivity from the Leydig cells of the testosterone-implanted animals only reflects a reduced cellular content (or storage) of this peptide but not its synthesis or release in response to hCG. Furthermore this may have been compensated by an increased synthesis of VEGF (as suggested by stronger immunostaining) from the Sertoli cells. The answers to the above questions would have to come from studies examining the Leydig cell VEGF mRNA expression and peptide secretion in these animals. Nonetheless, it is possible to conclude that the hCG-induced endothelial cell proliferation in adult rat testes is critically dependent on the presence but not so much the functional state of the Leydig cells, and the involvement of Leydig cell-derived VEGF in this process cannot be entirely ruled out.

Besides VEGF, other inflammation mediators and/or angiogenic factors from pro-inflammatory cells may also participate in stimulating endothelial cell proliferation in the testis (Collin & Bergh, 1996). Indeed in the present study, there was an association between increased endothelial cell proliferation in response to hCG and an inflammation-like reaction as indicated indirectly by an increase in testis weight from the accumulation of testicular interstitial fluid. This could be observed in the testes of normal rats and rats that received testosterone implants, but not those animals treated with EDS to deplete the Leydig cells. At present, it remains unclear what inflammation mediators and/or other angiogenic factors are involved and whether they

are derived from Leydig cells, testicular macrophages or the infiltration of PMN leukocytes.

The present data also indicate that the spermatogenic activity within the seminiferous tubules or the mass of the tubules has no significant contribution towards determining the proliferative response of vascular endothelial cells to hCG stimulation. Such reasoning can be drawn from the studies of animals bearing the two doses of testosterone implants and from the EDS-treated group. In the 3 cm-implant group, testis weight fell by one-third due to inadequate hormonal support to maintain normal spermatogenic activity within the seminiferous tubules. Despite the loss of tubule mass, the hCG-induced endothelial cell proliferation remained insignificantly affected when compared with the 25 cm-implant group or the normal control. This contrasts strongly against the acute treatment by EDS when at 4-5 days after treatment, the proliferative response of endothelial cells to hCG stimulation completely disappears despite an insignificant fall in testis weight. Thus there is no fixed relationship between the levels of hCG-induced endothelial cell proliferation and the tubule mass or the level of spermatogenic activity within the tubular compartment.

As earlier pointed out, comparisons of endothelial cell proliferation per unit section area among different treatment groups and the control have to base on the assumption that the testis size remained little affected. However in this experiment, animals bearing the 3 cm testosterone implant had a reduced level of spermatogenesis due to inadequate hormonal support, and the shrinkage of the tubular compartment had exaggerated the value of PCNA-positive endothelial cell number per unit section area. As a method of adjustment, the number of PCNA-positive endotheial cells was instead related to per unit intertubular area.

As previously discussed in section 4.1, the testosterone response to hCG does not appear to be linked to the hCG-induced endothelial cell proliferation. This was again confirmed in the study of rats bearing the testosterone implants. In these animals, the testosterone response to hCG stimulation was compromised due to the chronic suppression of Leydig cells by the exogenous testosterone, and a small but significant increase in serum testosterone was observed only in the 25 cm-implant group. However, despite the large differences in circulating testosterone levels achieved in the two implanted groups, their stimulated increases in endothelial cell proliferation (when expressed as per unit intertubular area) were almost identical. These observations suggest that the hCG-induced endothelial cell proliferation is mediated by Leydig cell products other than steroids.

4.3 Role of testicular macrophages in hCG-induced endothelial cell proliferation in adult rat testis

hCG-induced endothelial cell proliferation (at 1 day and 2 days post-hCG) in adult rat testes was enhanced following activation of testicular macrophages by latex beads. The two treatments appear to synergize with each other since their combined actions resulted in nearly twice the sum of their individual effect in producing an increase in the density of PCNA-positive endothelial cells (per unit section area). The mechanism of such interaction remains unclear but it is possible that angiogenic factors from Leydig cells and activated macrophages (as reviewed in the 'Introduction') may synergize with each other in stimulating the proliferation of endothelial cells. It has been reported that cultured macrophages activated by latex beads phagocytosis are able to induce vascular proliferation (Polverini et al., 1977). In addition, testicular macrophages and Leydig cells are known to exhibit significant

structurally and functionally interaction (Miller, Bowman & Rowland, 1983; Bergh, 1985), and thus activated macrophages may potentiate the hCG-induced Leydig cell-mediated vascular changes through a paracrine mechanism. Kerr & Sharpe (1989) have used the same method of activating testicular macrophages by latex beads and reported that the inflammation-like reaction of the intertubular tissue associated with hCG administration is enhanced. Therefore it is also possible that the angiogenic response is potentiated by the higher levels of inflammation mediators originating from the macrophages and the infiltration of PMN leukocytes (Leibovich *et al.*, 1987; Sunderkotter *et al.*, 1991; Jackson *et al.*, 1997).

In the present study, testicular macrophages were activated by an intratesticular injection of polystyrene latex beads and the effect was studied 4-5 days after treatment. However, even under basal condition (in the absence of hCG stimulation) and with the injection of sterile saline (in the sham-operated control), the procedure alone already induced an artifactual increase in the number of PCNA-positive endothelial cells. This could have resulted from the reaction to tissue damage caused by the passage of the needle, and would also explain why this artifactual stimulation appeared to subside with time after treatment.

Based on the results of the above study, it would appear that the hCG-induced endothelial cell proliferation will be compromised after the depletion of testicular macrophages. Also, this would be expected based on the fact that tissue macrophages are a source of angiogenic factors and pro-inflammatory mediators (Sunderkotter *et al.*, 1991) and its depletion has previously been shown to reduce the rate of angiogenesis during wound healing (Leibovich & Ross, 1975). However, in the present study, depletion of testicular macrophages by Cl_2MDP-lp treatment was found to have potentiated the effect of hCG in producing an even larger increase in the

number of PCNA-positive endothelial cells in the testicular vasculature. The same procedure has previously been shown to enhance the hCG-induced inflammation-like response (Bergh, Damber & van Rooijen, 1993). Indeed in the present study, many PMN leukocytes could still be found in the interstitium of the macrophage-depleted testes 48 hours post-hCG, whereas in normal testis, most of the PMN leukocytes would have disappeared and their number returned back to pre-treatment levels 32 hours post-hCG (Bergh *et al.*, 1986). As earlier proposed by Bergh & his coworkers (1993b), the loss of testicular macrophages may have removed an inhibitory influence that limits the development of the inflammation-like reaction induced by hCG stimulation. Secondary to the above changes, the prolonged infiltration and/or accumulation of PMN leukocytes may have provided an alternative source of angiogenic factors or inflammation mediators (Kibbey *et al.*, 1994; Ferrara, 1995; Gaudry *et al.*, 1997) to potentiate the endothelial cell proliferation caused by hCG. Nonetheless, since not the full complement of leukocyte subtypes (i.e. macrophages and PMN leukocytes) would be available, thus it is not surprising to find that testicular macrophage depletion is less effective when compared with testicular macrophage activation in potentiating the hCG-induced endothelial cell proliferation in the testicular vasculature.

In this study, although Cl_2MDP-lp (or PBS-lp for the sham-operated control) was also given by intratesticular injection, no artifactual increase in endothelial cell proliferation was observed under the basal state (without hCG stimulation). This could be related to the fact that the intratesticular injection was given at least 2 weeks prior to the tissue collection for the study of endothelial cell proliferation and any tissue damage or reaction would have already subsided after this period.

4.4 Testicular angiogenesis during postnatal development

The developmental changes in testicular angiogenesis during the postpartum life of rats from the age of 10- to 90-day old were examined using the methods of PCNA immunostaining to identify proliferating endothelial cells and ink perfusion to label the testicular blood vessels. Depending on the method of quantification and expression, different patterns of changes have emerged. Normalizing the number of PCNA-positive endothelial cells and blood vessels against per unit section area would not give an accurate picture of the changes due to the fact the intertubular area where the blood vessels are located does not constitute a fixed percentage of the total testis section area. During the development of the testis, the initiation and final establishment of spermatogenesis inside the seminiferous tubules has led to a rapid expansion in the size of the tubular compartment initially, before it gradually stabilizes to the level found in adult animals. As a result, there is an age-related decrease in the percentage of testis section area occupied by the intertubular tissues as illustrated in the present study. The difference in this value between immature and adult testes (>60 day-old) is not particularly marked and statistically higher percentages could only be demonstrated for animals at the age of 20-day old or younger. Similar findings have previously been reported based on the volume density of seminiferous tubules in rats (Gazdzik, et al., 1985; Gaytan *et al.*, 1986; Kerr, Risbridger & Knell, 1988). This value does not appear to change much during postnatal development although it forms a lesser proportion of the testis mainly prior to 20-day old, when compared with that in the adult. Thus in the present study, when the number of PCNA-positive endothelial cells or blood vessels was expressed over per unit section area, there is a trend for this value to decrease with the age of the animals.

When the number of PCNA-positive endothelial cells was normalized against per unit intertubular area, the maximum values were found in the testes of 10- to 30-day old rats. These data agree extremely well with earlier studies carried out using other methods of quantifying proliferating endothelial cells in rats, hamsters and mice. In an earlier study performed on Sprague Dawley rats, a marked increase in the total number of endothelial cells in the testis (as determined using the stereological method) was found between Day 7 and Day 28 post partum (Hardy, Zirkin & Ewing, 1989). When tritated thymidine was used to label the proliferating endothelial cells, high labelling index was found between Day 14 and Day 24 post partum. In the golden hamster, testicular angiogenesis as indicated subjectively by the morphological appearance of endothelial cell migration was found to be most conspicuous in the testes of 8- to 25-old animals (Mayerhofer & Bartke, 1990). In mice, the total number of endothelial cells as determined also using the stereological method, was found to increase significantly from the age of Day 11 to Day 28 (Vergouwen *et al.*, 1993).

Following a marked increase in the density of PCNA-positive endothelial cells (per unit intertubular area) in the testes of 10-30 days old rats, the blood vessel density (expressed also on the same basis of per unit intertubular area) was found to reach its second peak in 50-day old rats. The first peak was found in 10-day old rats and this could be related to a very small size of the testis at this age. After spermatogenesis and Leydig cell development begin at the age of 19-20 days and 15-18 days post partum, respectively, both the tubular and intertubular compartments rapidly expand in size and this may explain why the blood vessel density in the testis actually fell in animals between the age of 20- and 40-day old. The marked increase in density of PCNA-positive endothelial cells was followed later on by a peak of blood

vessel density since time is required for new blood vessels to be laid down after an earlier step involving endothelial cell proliferation. In the present study, using ink perfusion followed by image analysis, it was not possible to establish whether the increase in vessel numbers is due to capillaries, venules or arterioles. Nevertheless, the results appear to agree chronologically with the onset time for the development of intertubular and peritubular capillary network at the age of 20-35 days as revealed using an angiographic method (Kornamo, 1967a). The cause for a decrease in testicular blood vessel density in animals after the age of 50-day old remains unclear. Whether this truly represents a regression of blood vessels or only the density was reduced relative to changes in tissue volume/area will have to await further investigation.

5. References

Au, C.L., Chung, H.S., Pang, H.C. & Chan, L.F. (1996) Human chorionic gonadotrophin (hCG)-induced endothelial cell proliferation in adult rat testes and its dependence on the presence of Leydig cells. *J Physiol* 497, 71P.

Au, C.L., Chan, L.F., Wan, W.C. & Lee, W.M. (1997) Localization of vascular endothelial growth factor (VEGF) in rodent testes. *Int J Androl* 20, 72. (Abstract)

Auerbach, R., Auerbach, W. & Polakowski, I. (1991) Assays of angiogenesis: A review. *Pharmacol Ther* 51, 1-11.

Auerbach, W. & Auerbach, R. (1994) Angiogenesis inhibition: a review. *Pharmacol Ther* 63, 265-311.

Augustin, H.G., Braun, K., Telemenakis, I., Modlich, U. & Kuhn W. (1995) Ovarian angiogenesis - phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. *Am J Pathol* 147: 339-351.

Bancroft, J.D. & Stevens, A. (1996) Theory and Practice of Histological Techniques. 4th Edition, Churchill Livingstone : London.

Beck, L. Jr. & D'more P.A. (1997) Vascular development: cellular and molecular regulation. *FASEB J* 11, 365-373.

Bergh, A. (1985) Effect of crpytorchidism on the morphology of testicular macrophages: evidence for a Leydig cell-macrophage interaction in the rat testis. *Int J Androl* 8, 86-96.

Bergh, A., Widmark, A., Damber, J.-E. & Cajander, S. (1986) Are leukocytes involved in the human chorionic gonadotrophin-induced increase in testicular vascular permeability? *Endocrinology* 119, 586-590.

Bergh, A., Rooth, P., Widmark, A. & Damber, J.-E. (1987) Treatment of rats with hCG induces inflammation-like changes in the testicular microcirculation. *J Reprod Fertil* 79, 135-143.

Bergh, A., Damber, J.-E. & Widmark, A. (1988) Hormonal control of testicular blood flow, microcirculation and vascular permeability. In: *The Molecular and Cellular Endocrinology of the Testis*, Cooke, B.A. & Sharpe, R.M. (eds.), Serono Symposia Publication, Vol.50. Raven Press: New York, pp. 123-134.

Bergh, A., Damber, J.-E. & Widmark, A. (1990) A physiological increase in LH may influence vascular permeability in the rat testis. *J Reprod Fertil* 89, 23-31.

Bergh, A. & Damber, J.-E. (1993) Vasscular controls in testicular physiology. In: *Molecular Biology of the Male Reproductive System*, de Krester, D.M. (ed.), New York: Academic Press. pp. 439-468.

Bergh, A., Damber, J.-E. & van Rooijen, N. (1993a) Liposome-mediated macrophage depletion: an experimental approach to study the role of testicular macrophages in the rat. *J Endocrinol* 136, 407-413.

Bergh, A., Damber, J.-E. & van Rooijen, N. (1993b) The human chorionic gonadotrophin-induced inflammation-like response is enhanced in macrophage-depleted rat testes. *J Endocrinol* 136, 415-420.

Bergh, A., Damber, J.-E. & Hjertkvist, M. (1996) Human chorionic gonadotrophin-induced testicular inflammation may be related to increased sensitivity to interleukin-1. *Int J Androl* 19, 229-236.

Bicknell, R., Lewis, C.E. & Ferrara, N. (1997) Introduction. In: *Tumour Angiogenesis*, Bicknell, R., Lewis, C.E. & Ferrara, N. (eds.), Oxford University Press: Oxford, pp. 1-3.

Breier, G., Damert, A., Plate, K.H. & Risau, W. (1997) Angiogenesis in embryos and ischemic diseases. *Thromb Haemost* 78, 678-683.

Brown, L.F., Berse, B., Jackman, R.W., Tognazzi, K., Guidi, A.J., Dvorak, H.F., Senger, D.R., Connolly, J.L. & Schnitt, S.J. (1995) Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol* 26, 86-91.

Charnock-Jones, D.S., Sharkey, A.M., Rajput-Williams, J., Burch, D., Schofield, J.P., Fountain, S.A., Boocock, C.A. & Smith, S.K. (1993) Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. *Biol Reprod* 48, 1120-1128.

Cockerill, G. W., Gamble, J.R. & Vadas, M. A. (1995) Angiogenesis: Models and modulators. *Int Rev Cytol* 159, 113-160.

Collin, O. & Bergh, A. (1996) Leydig cells secrete factors which increase vascular permeability and endothelial cell proliferation. *Int J Androl* 19, 221-228.

Cooper, J.C., Sharkey, A.M., MaLaren, J., Charnock-Jones, D.S. & Smith, S.K. (1995) Localization of vascular endothelial growth factor and its receptor, flt, in human placenta and decidua by immunohistochemistry. *J Reprod Fertil* 105, 205-213.

Cullinan-Bove, K. & Koos, R.D. (1993) Vascular endothelial growth factor / vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology* 133, 829-837.

Das, S.K., Chakraborty, I., Wang, J., Dey, S.K. & Hoffman, L.H. (1997) Expression of vascular endothelial growth factor (VEGF) and VEGF-receptor messenger

ribonucleic acids in the peri-implantation rabbit uterus. *Biol Reprod* 56, 1390-1399.

Desjardins, C. (1989) The microcirculation of the testis. *Ann N Y Acad Sci* 564, 243-249.

Desjardins, C. (1993) Design and function of the microcirculation. In: *Cell and Molecular Biology of the Testis*, Desjardins, C. & Ewing, L.L.(eds.), New York: Oxford University Press. pp. 127-136.

Dissen, G.A., Lara, H.E., Fahrenbach, W.H., Costa, M.E. & Ojeda, S.R. (1994) Immature rat ovaries become revascularized rapidly after autotransplantation and show a gonadotropin-dependent increase in angiogenic factor gene expression *Endocrinology* 134: 1146-1154

Dvorak, H.F., Brown, L.F., Detmar, M. & Dvorak, A.M. (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146, 1029-1039.

Ergun, S., Kilic, N., Fiedler, W. & Mukhopadhyay, A.K. (1997) Vasuclar endothelial growth factor and its receptors in normal human testicular tissue. *Mol Cell Endocrinol* 131, 9-20.

Espey, L.L. (1980) Ovulation as an inflammatory reaction - a hypothesis. *Biol Reprod* 22, 73-106.

Ferenczy, A., Bertrand, G. & Gelfand M.M. (1979) Proliferation kinetics of human endometrium during the normal menstrual cycle. *Am J Obstet Gynecol* 133, 859-867.

Ferrara, N., Houck K., Jakeman, L. & Leung, D.W. (1992) Molecular and biological properties of the vascular endothelial growth factor family of proteins *Endocr Rev* 13, 18-32.

Ferrara, N. (1995) Leukocyte adhesion. Missing link in angiogenesis. *Nature* 376, 467.

Ferrara, N. (1996) Vascular endothelial growth factor. *Eur J Cancer* 32A, 2413-2422.

Ferrara, N. & Davis-Smyth, T. (1997) The biology of vascular endothelial growth factor. *Endocr Rev* 18, 4-25.

Ferrara, N., Chen, H., Davis-Smyth, T., Gerber, H.P., Nguyen, T.N., Peers, D., Chisholm, V., Hillan, K.J., Schwall, R.H. (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nat Med* 4, 336-340.

Findlay, J.K. (1986) Angiogenesis in reproductive tissues. *J Endocrinol* 111, 357-366.

Folkman, J. & Haudenschild, C. (1980) Angiogenesis in vitro. *Nature* 288, 551-556.

Folkman, J. & Klagsbrun, M. (1987) Angiogenic factors. *Science* 235, 442-443.

Folkman, J. (1995a) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1, 27-31.

Folkman, J. (1995b) clinical applications of research on angiogenesis. *N Engl J Med* 333, 1757-1763.

Free, M.J. (1977) Blood supply to the testis and its role in local exchange and transport of hormones. In: *The Testis*, Vol III, Johnson, A.D. & Gomes, W.R.(eds.), New York: Academic Press. pp. 39-90.

Gasparini, G. (1996) Angiogenesis research up to 1996. A commentary on the state of art and suggestions for future studies. *Eur J Cancer* 32A, 2379-2385.

Gaudry, M., Bregerie, O., Andrieu, V., El Benna, J., Pocidalo, M.A. & Hakim, J. (1997) Intracellular pool of vascular endothelial growth factor in human neutrophils. *Blood* 90, 4153-4161.

Gaytan, F., Lucena M.C., Munoz, E. & Paniagua, R. (1986) Morphometric aspects of rat testis development. *J Anat* 145, 155-159.

Gazdzik, T., Kaminski, M., Wagiel, J. & Kowalska, G. (1985) Morphometry of the rat testis in postnatal period. I. Physiological conditions. *Folia Morphol* 44, 20-23.

Gerdes, U., Gafvels, M., Bergh, A. & Cajander, S. (1992) Localized increases in ovarian vascular permeability and leucocyte accumulation after induced ovulation in rabbits. *J Reprod Fert* 95, 539-550.

Giudice, L.C. (1994) Growth factors and growth modulators in human uterine endometrium: their potential relevance to reproductive medicine. *Fertil Steril* 61, 1-17.

Gnessi, L., Fabbri, A. & Spera, G. (1997) Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. *Endocr Rev* 18, 541-608.

Goodger, A.M. & Rogers, P.A. (1993) Uterine endothelial cell proliferation before and after embryo implantation in rats. *J Reprod Fertil* 99, 451-457.

Gordon, J.D., Shifren, J.L., Foulk, R.A., Taylor, R.N. & Jaffe, R.B. (1995) Angiogenesis in the human female reproductive tract. *Obstet Gynecol Surv* 50, 688-697.

Gordon, J.D., Mesiano, S., Zaloudek, C.J. & Jaffe, R.B. (1996) Vascular endothelial growth factor localization in human ovary and fallopian tubes: possible role in

reproductive function and ovarian cyst formation. *J Clin Endocrinol Metab* 81, 353-359.

Hanahan, D. & Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353-364.

Hanahan, D. (1997) Signaling vascular morphogenesis and maintenance. *Science* 277, 48-50.

Hardy, M.P., Zirkin, B.R. & Ewing, L.L. (1989) Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat. *Endocrinology* 124, 762-770.

Heron, M.I., Kolar, F., Papousek, F. & Rakusan, K. (1997) Early and late effect of neonatal hypo- and hyperthyroidism on coronary capillary geometry and long-term heart function in rat. *Cardiovasc Res* 33, 230-240.

Hjertkvist, M., Lackgren, G., Ploen, L. & Bergh, A. (1993) Does HCG treatment induce inflammation-like changes in undescended testes in boys? *J Pediatr Surg* 28, 254-258.

Hodgson, Y.M. & de Kretser, D.M. (1982) Serum testosterone response to single injection of hCG ovine-LH and LHRH in male rats. *Int J Androl* 5, 81-91.

Hodgson, Y.M. & de Kretser, D.M. (1984) Acute responses of Leydig cells to hCG: evidence for early hypertrophy of Leydig cells. *Mol Cell Endocr* 35, 75-82.

Hyder, S.M., Stancel, G.M., Chiappetta, C., Murthy, L., Boettger-Tong, H.L. & Makela, S. (1996) Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. *Cancer Res* 56, 3954-3960.

Iruela-Arispe, M.L. & Dvorak, H.F. (1997) Angiogenesis: a dynamic balance of stimulators and inhibitors. *Thromb Haemost* 78, 672-677.

Jackson C.M. & Jackson H. (1984) Comparative protective action of gonadotrophins and testosterone against the anti-spermatogenic action of ethane dimethanesulphonate. *J Reprod Fertil* 71, 393-401.

Jackson, J.R., Seed, M.P., Kircher, C.H., Willoughby, D.A. & Winkler, J.D. (1997) The coindependence of angiogenesis and chronic inflammation. *FASEB J* 11, 457-465.

Jain, R. K., Schlenger, K., Hockel, M. & Yuan F. (1997) Quantitative angiogenesis assays: progress and problems. *Nat Med* 3, 1203-1208.

Kamat, B.R., Brown, L.F., Manseau, E.J., Senger, D.R. & Dvorak, H.F. (1995) Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. *Am J Pathol* 146, 157-165.

Kern, S., Robertson, S.A., Mau, V.J. & Maddocks, S. (1995) Cytokine secretion by macrophages in the rat testis. *Biol Reprod* 53, 1407-1416

Kerr, J.B., Risbridger, G.P. & Knell, C.M. (1988) Stimulation of interstitial cell growth after selective destruction of foetal Leydig cells in the testis of postnatal rats. *Cell Tissue Res* 252, 89-98.

Kerr, J.B. & Sharpe, R.M. (1989) Macrophage activation enhances the human chorionic gonadotrophin-induced disruption of spermatogenesis in the rat. *J Endocrinol* 121, 285-292.

Kibbey, M.C., Corcoran, M.L., Wahl, L.M. & Kleinman H.K. (1994) Laminin SIKVAV peptide-induced angiogenesis in vivo is potentiated by neutrophils. *J Cell Physiol* 160, 185-193.

Koos, R.D. (1995) Increased expression of vascular endothelial growth factor / permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. *Biol Reprod* 52: 1426-1435.

Koos, R.D. & LeMaire W.J. (1983) Evidence for an angiogenic factor from rat follicles. In *Factors Regulating Ovarian Function*, Greenwald, G.S. & Terranova, P.F. (eds.), Raven Press: New York, pp. 191-195.

Kormano, M. (1967a) An angiographic study of the testicular vasculature in the postnatal rat. *Z Anat Entwicklungsgesch* 126, 138-153.

Kormano, M. (1967b) Dye permeability and alkaline phosphatase activity of testicular capillaries in the postnatal rat. *Histochemie* 9, 327-338.

Kuroda, M., Oka, T., Oka, Y., Yamochi, T., Ohtsubo, K., Mori, S., Watanabe, T. & Machinami, R. (1995) Colocalization of vascular endothelial growth factor (vascular permeability factor) and insulin in pancreatic islet cells. *J Clin Endocrinol Metab* 80, 3196-3200.

Laitinen, M., Ristimaki, A., Honkasalo, M., Narko, K., Paavonen, K. & Ritvos, O. (1997) Differential hormonal regulation of vascular endothelial growth factors VEGF, VEGF-B, and VEGF-C messenger ribonucleic acid levels in cultured human granulosa-luteal cells. *Endocrinology* 138, 4748-4756.

Leek, R.D., Lewis C.E. & Harris, A.L. (1997) The role of macrophages in tumor angiogenesis. In: *Tumour Angiogenesis*, Bicknell, R., Lewis, C.E. & Ferrara, N. (eds.), Oxford University Press. pp. 81-99.

Leibovich, S.J., Polverin, P.J., Shepard, H.M., Wiseman, D.M., Shively, V. & Nuseir, N. (1987) Macrophage-induced angiogenesis is mediated by tumour necrosis factor-α. *Nature* 329, 630-632.

Leibovich, S.J. & Ross, R. (1975) The role of the macrophage in wound repair: a study with hydrocortisone and anti-macrophage serum. *Am J Pathol* 78, 71-100.

Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V. & Ferrara, N. (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246, 1306-1309.

Linden, M.D., Torres, F.X., Kubus, J. & Zarbo, R.J. (1992) Clinical application of morphologic and immunocytochemical assesments of cell proliferation. *Am J Clin Pathol* 97, s4-s13.

Maddox, P.H. & Jenkins, D. (1987) 3-aminopropyltriethoxysilane (APES): a new advance in section adhesion. *J Clin Pathol* 40, 1256-1260.

Mayerhofer, A. & Bartke, A. (1990) Developing testicular microvasculature in the golden hamster, *Mesocricetus auratus*: a model for angiogenesis under physiological conditions. *Acta Anat* 139, 78-85.

McLachlan, R.I., Wreford, N.G., Meachem, S.J., de Kretser, D.M. & Robertson, D.M. (1994) Effects of testosterone on spermatogenic cell populations in the adult rat. *Biol Reprod* 51: 945-955.

McLaren, J., Prentice, A., Charnock-Jones, D. S., Millican, S. A., Muller, K. H., Sharkey, A. M. & Smith, S. K. (1996) Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. *J Clin Invest* 98, 482-489.

Miller, S.C., Bowman, B.M. & Rowland, H.G. (1983) Structure, cytochemistry, endocytic activity and immunoglobulin (Fc) receptors of rat testicular interstitial-tissue macrophages. *Am J Anat* 168, 1-13.

Modlich, U., Kaup, F.J. & Augustin, H.G. (1996) Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involves endothelial cell detachment and vessel occlusion. *Lab Invest* 74, 771-780.

Nanji, A.A., Tahan, S.R., Wei, Y. & Sadrzadeh, S.M. (1994) Hepatic sinusoidal endothelial cell G1/S arrest correlates with severity of alcoholic liver injury in the rat. *Gastroenterology* 107, 818-823.

Nanji, A.A.& Tahan, S.R. (1996) Association between endothelial cell proliferation and pathologic changes in experimental alcoholic liver disease. *Toxicol Appl Pharmacol* 140, 101-107.

Neulen, J., Yan, Z., Raczek, S., Weindel, K., Kdck, C., Weich, H.A., Marme, D. & Breckwoldt, M. (1995) Human chorionic gonadotropin-dependent expression of vascular endothelial growth factor/vascular permeability factor in human granulosa cells: importance in ovarian hyperstimulation syndrome. *J Clin Endocrinol Metab* 80: 1967-1971.

Niemi, M., Sharpe, R.M. & Brown, W.R.A. (1986) Macrophages in the interstitial tissue of the rat testis. *Cell Tissue Res* 243: 337-344.

Norrby, K. (1997) Angiogenesis: new aspects relating to its initiation and control. *APMIS* 105, 417-437.

Pepper, M.S. (1997) Manipulating angiogenesis. From basic science to the bedside. *Arterioscler Thromb Vasc Biol* 17, 605-619.

Phillips, H.S., Hains, J., Leung, D.W. & Ferrara, N. (1990) Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* 127, 965-967.

Polverini, P.J., Cotran, P.S., Gimbrone, M.A.Jr & Unanue, E.R. (1977) Activated macrophages induce vascular proliferation. *Nature* 269, 804-806.

Polverini, P.J. (1995) The pathophysiology of angiogenesis. *Crit Rev Oral Biol Med* 6, 230-247.

Ravindranath, N., Little-Ihrig, L., Phillips, H. S., Ferrara, N. & Zeleznik, A. J. (1992) Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. *Endocrinology* 131, 254-260.

Reynolds, L.P., Killilea, S.D. & Redmer, D.A. (1992) Angiogenesis in the female reproductive system. *FASEB J* 6, 886-892.

Risau, W. (1997) Mechanisms of angiogenesis. *Nature* 386, 671-674.

Robaire, B., Ewing, L.L., Irby, D.C. & Desjardins C. (1979) Interactions of testosterone and estradiol-17β on the reproductive tract of the male rat. *Biol Reprod* 21: 455-463.

Sato, E., Ishibashi, T. & Koide, S.S. (1982) Induction of blood vessel formation by ovarian extracts from mice injected with gonadotropins. *Experientia* 38, 1248-1249.

Sato, K., Yamazaki, K., Shizume, K., Kanaji, Y., Obara, T., Ohsumi, K., Demura, H., Yamaguchi, S. & Shibuya, M. (1995) Stimulation by thyroid-stimulating hormone and Grave's immunoglobulin G of vascular endothelial growth factor mRNA expression in human thyroid follicles *in vitro* and *flt* mRNA expression in the rat thyroid *in vivo*. *J Clin Invest* 96, 1295-1302.

Schiffenbauer, Y.S., Abramovitch, R., Meir, G., Nevo, N., Holzinger, M., Itin, A., Keshet, E. & Neeman, M. (1997) Loss of ovarian function promotes angiogenesis in human ovarian carcinoma. *Proc Natl Acad Sci U S A* 94, 13203-13208.

Setchell, B.P. & Sharpe, R.M. (1981) The effect of human chorionic gonadotrophin on capillary permeability, extracellular fluid volume and flow of lymph and blood in the testis of rats. *J Endocrinol* 91, 245-254.

Setchell, B.P., Maddocks, S. & Brooks, D.E. (1994) Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: *The Physiology Reproduction*, 2nd Edition. Knobil, & Neill, J.D. (eds.) New York, Raven Press. pp. 1063-1175.

Sharpe, R.M. (1979) Gonadotrophin-induced accumulation of 'interstitial fluid' in the rat testis. *J Reprod Fertil* 55, 365-371.

Sharpe, R.M. & Cooper, I. (1983) Testicular interstitial fluid as a monitor for changes in the intratesticular environment in the rat. *J Reprod Fertil* 69, 125-135.

Shweiki, D., Itin, A., Neufeld, G., Gitay-Goren, H. & Keshet, E. (1993) Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest* 91, 2235-2243.

Soh, E.Y., Sobhi, S.A., Wong, M.G., Meng, Y.G., Siperstein, A.E., Clark, O.H. & Duh, Q.Y. (1996) Thyroid-stimulating hormone promotes the secretion of vascular endothelial growth factor in thyroid cancer cell lines. *Surgery* 120, 944-947.

Soini, Y., Pollanen, R., Kemppainen, S., Paakko, P. & Lehto, V.P. (1996) The association of vascular proliferation with HPV status and epithelial PCNA positivity in cervical intraepithelial lesions. *APMIS* 104, 183-190.

Sun, Y.T., Irby, D.C., Robertson, D.M. & de Kretser, D.M. (1979) The effects of exogenously administered testosterone on spermatogenesis in intact and hypophysectomized rats. *Endocrinology* 125: 1000-1010.

Sunderkotter, C., Goeveler, M., Schulze-Osthoff, K., Bhardwaj, R. & Sorg, C. (1991) Macrophage-derived angiogenesis factors. *Pharmacol Ther* 51, 195-216.

Taraska, T., Reynolds, L.P. & Redmer, D.A. (1989) *In vitro* secretion of angiogenic activity by ovine follicles. In *Growth Factors and the Ovary*, Hirshfield, A.N. (ed.), Plenum Press: New York, pp. 267-272.

Torry, D.S., Holt, V.J., Keenan, J.A., Harris, G., Caudle, M.R. & Torry, R.J. (1996) Vascular endothelial growth factor expression in cycling human endometrium. *Fertil Steril* 66, 72-80.

van Rooijen, N. (1989) The liposome-mediated macrophage 'suicide' technique. *J Immunol Methods* 124, 1-6.

van Vliet, J., Rommerts, F.F.G., de Rooij, D.G., Buwalda, G. & Wensing, C.J.G. (1988) Reduction of testicular blood flow and focal degeneration of tissue in the rat after administration of human chorionic gonadotrophin. *J Endocrinol* 117, 51-57.

Vergouwen, R.P.F.A., Jacobs, S.G., Huiskamp, R., Davids, J.A. & de Rooij, D.G. (1991) Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. *J Reprod Fertil* 93, 233-243.

Vergouwen, R.P.F.A., Huiskamp, R., Bars, R.J., Roepers-Gajadien, H.L., Davids, J.A. & de Rooij, D.G. (1993) Postnatal development of testicular cell populations in mice. *J Reprod Fertil* 99, 479-485

Viglietto, G., Romano, A., Manzo, G., Chiappetta, G., Paoletti, I., Califano, D., Galati, M.G., Mauriello, V., Bruni, P., Lago, C.T., Fusco, A. & Persico, M.G. (1997) Upregulation of the angiogenic factors PlGF, VEGF and their receptors (Flt-1, Flk-1/KDR) by TSH in cultured thyrocytes and in the thyroid gland of

thiouracil-fed rats suggest a TSH-dependent paracrine mechanism for goiter hypervascularization. *Oncogene* 15, 2687-2698.

Widmark, A., Bergh, A., Damber, J.-E. & Smedegard, G. (1987) Leucocytes mediate the hCG-induced increase in testicular venular permeability. *Mol Cell Endocrinol* 53, 25-31

Widmark, A., Damber, J.-E. & Bergh, A. (1986) Relationship between human chorionic gonadotrophin-induced changes in testicular microcirculation and the formation of testicular interstitial fluid. *J Endocrinol* 109, 419-425.

Widmark, A., Damber, J.-E. & Bergh, A. (1989) High and low doses of luteinizing hormone induce different changes in testicular microcirculation. *Acta Endocrinol* 121, 621-627.

William, R.F. (1949) Some responses of living blood vessels and connective tissue to testicular grafts in rabbits. *Anat Rec* 104, 147-161.

Woosley, J.T. (1991) Measuring cell proliferation. *Arch Pathol Lab Med* 115, 555-557.

Yamamoto, S., Konishi, I., Tsuruta, Y., Nanbu, K., Mandai, M., Kuroda, H., Matsushita, K., Hamid, A.A., Yura, Y. & Mori, T. (1997) Expression of vascular endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary. *Gynecol Endocrinol* 11, 371-381.